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(54) Title: FATTY ACID 9–HYDROPEROXIDE LYASE NUCLEIC ACID SEQUENCES

(57) Abstract

This invention relates to 9–Hydroperoxide Lyase or 9–HPO lyase enzymes. DNA constructs useful for the expression of a plant HPO lyase in a cell are described. Furthermore, DNA constructs useful for the antisense expression of a 9–HPO lyase in a plant cell are described. Such constructs will contain a DNA sequence encoding the 9–HPO lyase of interest under the control of regulatory elements capable of preferentially directing the expression of the 9–HPO lyase in plant tissue, when such a construct is expressed in a trangenic plant. This invention also relates to methods of using a DNA sequence encoding a 9–HPO lyase for the modification of the volatile aldehydes in plant tissues, as well as for methods of increasing disease resistance in a plant. 9–HPO Lyase sequences exemplified herein are obtained from *Arabidopsis*.

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Fatty Acid 9-Hydroperoxide Lyase Nucleic Acid Sequences

INTRODUCTION

Technical Field

This invention relates to the application of genetic engineering techniques to plants. More specifically, the invention relates to plant hydroperoxide lyase sequences and methods for the use of such sequences.

Background

With the development of genetic engineering techniques, it is now possible to transfer genes from a variety of organisms into the genome of a large number of different plant species. This process has many advantages over plant breeding techniques, as genes may now be transferred from one plant species to another plant species, rather than simply from a plant to the same, or different, but closely related, species.

Degradation of polyunsaturated fatty acids starts by the oxygenation at cis-cis double bonds of polyunsaturated fatty acids. This reaction is catalyzed by lipoxygenase (EC 1.13.11.12) enzymes which are present in plants, animals and microorganisms. The oxygenated products, called fatty acid hydroperoxides, are precursors for many important hormones (e.g. lipoxins, jasmonic acid, traumatic acid) and flavor/fragrance molecules (e.g. cis-3-hexenol, 1-octen-3-ol) in plants.

Compounds, such as jasmonic acid, are produced from hydroperoxides, such as 13-hydroperoxylinolenic acid, via an allene oxide synthase (referred to as AOS) and an allene oxide cyclase (referred to as ACS)-dependent pathway. Jasmonic acid is involved in stress and disease resistance signaling responses via the octadecanoid pathway. 13-hydroperoxylinolenic can also be catabolized by peroxygenases to form cutin monomers. Alternatively, 13-hydroperoxylinolenic

can be catabolyzed by hydroperoxide lyase eventually forming volatile aldehydes and traumatic acid.

Fatty acid hydroperoxide lyase (HPO lyase) catalyzes the cleavage of carbon-carbon bonds in polyunsaturated fatty acid hydroperoxides to produce short-chain aldehydes and ω-oxo-acids (Vick, *et al.* (1976) *Plant Physiol.* 57:780-788). The products of lysis of fatty acid hydroperoxides, such as short-chain volatile aldehydes are common in plant species. The aromas/flavors produced are a function of the specific products produced by the lysis of specific fatty acid hydroperoxides. For example, some short-chain volatile aldehydes contribute to the "green notes" in a wide variety of plant leaves, vegetables and fruits. "Green notes" are volatile molecules that contribute to the organoleptic qualities of flavor and fragrance of edible plant tissues. These qualities are often referred to as grassy, or "green" characteristics. Other short-chain volatile aldehydes, such as (3Z, 6Z)-nonadienol produced by the lysis of fatty acid 9-hydroperoxide by a fatty acid 9-hydroperoxide lyase (9-HPO lyase or 9-HPOL), contribute a melon aroma and/or a melon flavor, or sometimes referred to as a "melon" or "fresh" characteristic, to fruits and vegetables. Such characteristics are important to industries concerned with fragrances and flavorings.

Furthermore, short-chain aldehydes are also thought to be involved in disease resistance. For example, Croft, *et al* ((1993) *Plant Physiol.* 101:13-24) recently reported that (3*Z*)-hexenol and (2*E*)-hexenal levels increased during a hypersensitive-response in kidney bean plants. In addition, they also demonstrated that (2*E*)-hexenal is an effective antibacterial agent.

The characterization of hydroperoxide (also referred to as HPO lyase or HPOL) is useful for the further study of plant fatty acid metabolism systems and for the development of transgenic plant with increased organoleptic properties, including aromas and flavors. Studies of plant mechanisms may provide means to further enhance, control, modify, or otherwise alter the organoleptic qualities of edible plant

tissues. Furthermore, the elucidation of the physiological roles of HPO lyase and it's products may be useful for the further study of disease resistance responses, such as the HR response. Of particular interest are the nucleic acid sequences of genes encoding proteins which may be useful for applications in genetic engineering.

Relevant Literature

Shibata, *et al.* (1995) *Plant Cell Physiol* 36:147-156 reports the purification of a HPO lyase from green bell pepper. Matsui, *et al.* (1996) *FEBS Letters* 394:21-24 reports the cloning of a HPO lyase from bell pepper and the protein encoded by the HPO lyase sequence resembles a cytochrome P450.

SUMMARY OF THE INVENTION

The present invention provides nucleic acid sequences encoding for proteins which catalyze the formation of short-chain aldehydes and oxo-acids from 9-hydroperoxide fatty acids. Such proteins are referred to herein as 9-hydroperoxide lyases or 9-HPO lyase.

By this invention, nucleic acid sequences encoding plant 9-HPO lyase may now be characterized with respect to enzyme activity.

Thus, this invention encompasses 9-HPO lyase nucleic acid sequences and the corresponding amino acid sequences, and the use of these nucleic acid sequences in the preparation of oligonucleotides containing 9-HPO lyase encoding sequences for analysis and recovery of plant 9-HPO lyase gene sequences. The 9-HPO lyase encoding sequence may encode a complete or partial sequence depending upon the intended use. All or a portion of the genomic sequence, or cDNA sequence, is intended.

Of special interest are recombinant DNA constructs which provide for transcription or transcription and translation (expression) of the 9-HPO lyase sequence in a host cell. In particular, constructs which are capable of transcription or transcription and translation in plant host cells are preferred. For some applications a reduction in 9-HPO lyase

may be desired. Thus, recombinant constructs may be designed having the plant 9-HPO lyase sequences in a reverse orientation for expression of an anti-sense sequence or use of co-suppression, also known as "transwitch", constructs may be useful. Such constructs may contain a variety of regulatory regions including transcriptional initiation regions obtained from genes preferentially expressed in plant seed tissue. For some uses, it may be desired to use the transcriptional and translational initiation regions of the 9-HPO lyase gene either with the 9-HPO lyase encoding sequence or to direct the transcription and translation of a heterologous sequence.

In yet a different aspect, this invention relates to a method for producing a 9-HPO lyase in a host cell or progeny thereof via the expression of a construct in the cell. Cells containing the 9-HPO lyase as a result of the production of the 9-HPO lyase encoding sequence are also contemplated herein.

In addition, methods for modifying the volatile aldehyde content in plant tissues, especially fruit and leaf tissue, as well as methods for producing transgenic plants with increased disease resistance are contemplated.

Also considered in this invention are the modified plants, tissues and aldehyde compositions obtained by expression of the 9-HPO lyase sequence and protein of this invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the complete nucleotide sequence of the *Arabidopsis* HPO lyase.

Figure 2 shows a comparison of the amino acid sequences of the bell pepper HPO lyase and the *Arabidopsis* HPO lyase-like sequence.

Figure 3 shows a comparison of the amino acid sequences of the *Arabidopsis* allene oxide synthase and the *Arabidopsis* HPO lyase-like sequence.

Figure 4 shows the complete nucleotide sequence of the tomato HPO lyase.

Figure 5 shows the complete nucleotide sequence of the cucumber allene oxide synthase.

Figure 6 shows the complete nucleotide sequence of the cucumber 9-Hydroperoxide Lyase.

Figure 7 shows the amino acid sequence alignment between the bell pepper, banana, and *Arabidopsis* HPO lyase, with the highly conserved peptide sequences highlighted.

Figure 8 Provides the percent similarity in the upper right corner and the percent divergence in the lower right corner for the nucleotide sequences (Figure 8A) and amino acid sequences (Figure 8A) of the bell pepper HPOL (CaHPOL), tomato fruit HPOL (LeHPOL), cucumber hypocotyl HPOL (CsC17HPOL, pseudogene), Arabidopsis inflorecence HPOL (AtHPOL), banana leaf HPOL (MsHPOL), cucumber hypocotyl 9-HPOL (Cs15HPOL), Guayule AOS (GuAOS), flaxseed AOS (LiAOS), and the Arabidopsis AOS (AtAOS).

Figure 9 shows the gas chromatography (GC) analysis of the cucumber 9-HPO lyase using linoleic acid 13-hydroperoxide (Figure 9A) and linoleic acid 9- hydroperoxide (figure 9B) substrates.

Figure 10 provides the results of the spectrophotometric assay of the cucumber 9-HPO lyase expressed from *E. coli* using linoleic acid 13-hydroperoxide and linoleic acid 9-hydroperoxide substrates.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, nucleotide sequences are provided which are capable of coding sequences of amino acids, such as, a protein, polypeptide or peptide, which demonstrate the ability to form short-chain aldehydes and oxo-acids from 9-hydroperoxide fatty acids under plant enzyme reactive conditions. By "enzyme reactive conditions" is meant that any necessary conditions are available in an environment (i.e., such factors as temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

In one embodiment of the present invention, nucleic acid sequences are provided which encode for 9-hydroperoxide lyase

(hereinafter referred to as 9-HPO lyase). Such nucleic acid sequences are demonstrated herein to encode enzymes which form (3Z,6Z)-nonadienal and 9-oxo-nonanoic acid from Linolenic acid 9-hydroperoxide or 9-hydroperoxy-(10E, 12Z, 15Z)-octadecadienoic acid.

In the examples provided below, a nucleic acid sequence from cucumber (*Cucumis sativus*) is identified from cDNA libraries made from total RNA isolated from cucumber hypocotyls. A full length coding sequence is obtained, and the product encoded by the full length sequence demonstrates activity towards the substrate linolenic acid 9-hydroperoxide to produce (3Z, 6Z)-nonadienal and 9-oxo-nonanoic acid.

In another embodiment of the present invention, expression constructs are provided which direct the expression of nucleic acid sequences encoding 9-HPO lyase in bacterial and plant tissues.

Of particular interest in the present invention, is the use of such expression constructs to produce transgenic plants with increased production of short-chain volatile aldehydes in plant fruits and tissues. Such volatile aldehydes are important constituents of the characteristic flavors of fruits, vegetables and green leaves. Thus, the 9-HPO lyase sequence of the present invention may be used in expression constructs to produce transgenic plants with improved organoleptic properties, in particular improved fruity (or "melon") note flavor and aroma characteristics.

The nucleic acid sequences of the present invention may also find use in expression constructs for the production of transgenic plants with increased resistance to various pathogens. Transgenic plants expressing the HPO lyase sequence of the present invention may exhibit an enhanced hypersensitive-reaction (HR response) in response to pathogen attack due to the increased production of aldehydes involved in the HR response, such as (3*Z*)-hexenal and (2*E*)-hexenal (Croft, *et al.* (1993) *Plant Physiol.* 101:13-24). Aldehydes, such as (2*E*)-hexenal, have also been shown to be effective anti-bacterial agents, further contributing to enhanced disease resistance (Croft, *et al.* (1993), *supra*).

Furthermore, these compounds may be involved in a general wounding response in plants.

Also of particular interest in the present invention is the use of 9-HPO lyase nucleic acid sequences in constructs to direct the expression of 9-HPO lyase in a prokaryotic and/or eukaryotic host cells for the production of flavorings and aromas.

To confirm the activity and specificity of proteins encoded by nucleic acid sequences as 9-HPO lyase enzymes, assays are performed on protein extracts of cultured bacterial cells expressing the protein. As described in more detail in the examples, cucumber 9-HPO lyase expression constructs are prepared to direct the expression of the 9-HPO lyase sequence in *E coli*. The expressed 9-HPO lyase enzymes demonstrate the ability to produce (3Z, 6Z)-nonoadienal and 9-oxo-nonanoic acid from linolenic acid 9-hydroperoxide or 9-hydroperoxy-(10E, 12Z, 15Z)-octadecadienoic acid by gc analysis.

The skilled artisan will recognize that several methods for the identification of additional sequences encoding 9-HPO lyase are available in the art.

For example, antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" HPO lyase sequences from a variety of plant sources. Typically, nucleic acid probes are labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal may be utilized. Polyclonal antibodies, although less specific, typically are more useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate systems that are commercially available. Examples of some of the available antibody detection systems are described by Oberfilder (*Focus* (1989) BRL Life Technol., Inc.,*11*:1-5).

In order to obtain additional HPO lyase sequences, a genomic or other appropriate library prepared from the candidate plant source of interest may be probed with

conserved sequences from one or more plant HPO lyase(s) to identify homologously related sequences. Positive clones may be analyzed by restriction enzyme digestion and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the HPO lyase gene from such plant source. Probes can also be considerably shorter than the entire sequence. Oligonucleotides may be used, for example, but should be at least about 10, preferably at least about 15, more preferably at least 20 nucleotides in length. When shorter length regions are used for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences can be identified. (*See*, Gould, *et al.*, *PNAS USA* (1989) *86*:1934-1938.)

When longer nucleic acid fragments are employed (>100 bp) as probes, especially when using complete or large cDNA sequences, one can still screen with moderately high stringencies (for example using 50% formamide at 37°C with minimal washing) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences. (For additional information regarding screening techniques see Beltz, *et al.*, *Meth. Enzymology* (1983) *100*:266-285).

Homologous sequences are found when there is an identity of sequence and may be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known HPO lyase and a candidate source. Conservative changes, such as Glu/Asp, Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may also be considered in determining sequence homology. Typically, a lengthy nucleic acid sequence may show as little as 50-60% sequence identity, and more preferably at least about 70% sequence identity, between the target sequence and the given plant HPO lyase of interest excluding any deletions which may be present, and still be considered related. Amino acid sequences are considered homologous by as little as 20% sequence identity between the two complete mature proteins.

(*See* generally, Doolittle, R.F., *OF URFS and ORFS* (University Science Books, CA, 1986.)

In addition, not only can sequences provided herein be used to identify homologous 9-hydroperoxide lyases, but the resulting sequences obtained therefrom may also provide a further method to obtain plant 9-hydroperoxide lyase sequences from other plant sources. In particular, PCR may be a useful technique to obtain related 9-HPO lyases from sequence data provided herein. One skilled in the art will be able to design oligonucleotide probes based upon sequence comparisons or regions of typically highly conserved sequence. Furthermore, additional sequences may be added to the 5' end of the primer to include restriction endonuclease cleavage sites to allow for convenient cloning of the PCR fragment into cloning vectors. Addition of such 5' "tails" is well known in the art, and do not interfere with amplification as mismatched nucleotides at the 5' end of primers are not required for amplification.

For example, as demonstrated in the examples that follow, amino acid sequence alignments between sequences encoding 13-hydroperoxy lyase from bell pepper (Matsui, *et al.* (1996) *FEBS Letters* 394:21-24), banana (European Patent Application, Publication Number EP 0 801 133 A2, the entirety of which is incorporated herein by reference) and *Arabidopsis* identify highly conserved peptide sequences. Synthetic oligonucleotide primers are constructed based on these peptide sequences and used in PCR reactions to amplify cDNAs from several plant tissue sources which are highly similar to known HPO lyase sequences. Complimentary DNA sequences are identified from tomato hypocotyl and fruit tissues and cucumber hypocotyl tissue which are highly homologous to the bell pepper 13-HPO lyase. However, a second cDNA sequence is obtained from the cucumber hypocotyl tissue which is divergent from the 13-HPO lyase sequences. Furthermore, the sequence shows a slightly higher similarity to allene oxide synthases.

The nucleotide sequence obtained from any method may be molecularly cloned into an appropriate vector for propagation of the DNA by methods known in the art. Many cloning vectors

are available commercially, and may be employed used with the nucleotide sequences of the present invention. For example, the DNA may be inserted into a pBluescript vector (Stratgene, La Jolla, CA). Typically, useful cloning vectors for bacterial use can comprise a selectable marker and a bacterial origin of replication derived from commercially available plasmids which are in turn derived from the well known cloning vector pBR322 (ATCC 37017). Further examples of cloning vectors include the pGEM vectors (Promega Biotec, Madison, WI). In addition, linear cloning vectors with single thymine ("T") overhangs are available for convenient cloning of PCR fragments amplified utilizing Taq DNA polymerase which usually adds an adenine ("A") to the end of the amplified fragment. Such vectors include pCR2.1 vector (Invitrogen, La Jolla, CA).

Cloned DNA sequences may be expressed in hosts to confirm HPO lyase activity. Techniques and expression vectors for expression in such hosts are well known in the art and are available from a wide variety of commercial sources. Examples of such vectors include pQE30 (Qiagen, Hilden, Germany). Furthermore, the sequences of the DNA coding regions can be optimized by gene resynthesis, based on codon usage, for maximum expression in particular hosts.

As described in more detail in the examples that follow, expression of the cucumber HPO lyase sequence in *E coli* demonstrates that the sequence encodes a 9-hydroperoxide lyase. This is the first reported sequence cloned, to the inventor's knowledge, encoding a hydroperoxide lyase with activity on linolenic acid 9-hydroperoxide substrates.

Once the nucleic acid sequence is obtained, the transcription, or transcription and translation (expression), of the 9-HPO lyase in a host cell is desired to produce a ready source of the enzyme and/or modify the composition of fatty acids and/or volatile compound found therein. Other useful applications may be found when the host cell is a plant host cell, *in vitro* and *in vivo*.

Nucleic acids (genomic DNA, plasmid DNA, cDNA, synthetic DNA, mRNA, etc.) encoding HPO lyase or amino acid sequences of the purified enzymes, which permit design of nucleic acid

probes facilitating the isolation of DNA coding sequences therefor, are known in the art and are available for use in the methods of the present invention. It is generally recognized to an artisan skilled in the field to which the present invention pertains that the nucleic acid sequences provided herein and the amino acid sequences derived therefrom may be used to isolate other potential HPO lyase genes from GenBank or other database using DNA and peptide search techniques generally known in the art.

In addition to the sequences described in the present invention, DNA coding sequences useful in the present invention can be derived from algae, fungi, bacteria, mammalian sources, plants, etc. Homology searches in existing databases using signature sequences corresponding to conserved nucleotide and amino acid sequences of 9-HPO lyase can be employed to isolate equivalent, related genes from other sources such as plants and microorganisms. Searches in EST databases can also be employed. Furthermore, the use of DNA sequences encoding enzymes functionally enzymatically equivalent to those disclosed herein, wherein such DNA sequences are degenerate equivalents of the nucleic acid sequences disclosed herein in accordance with the degeneracy of the genetic code, is also encompassed by the present invention. Demonstration of the functionality of coding sequences identified by any of these methods can be carried out by expression of such sequences in various expression systems employing various organisms.

Furthermore, constructs for the expression of the 9-HPO lyase sequences in plants are described. Also, the use of such 9-HPO lyase expression constructs for increasing aldehyde and oxo-acid production in plant tissues is contemplated in the present invention. The 9-HPO lyase expression constructs may be employed to produce a host plant having modified organoleptic properties. By organoleptic properties is meant as relating to qualities, such as taste, color, odor, and feel, of a substance, such as a food, that stimulate the sense organs. Furthermore, constructs may be prepared to direct the expression of the 9-HPO lyase sequence in a host cell to

provide for the production of aldehydes and oxo-acids for use in flavorings and fragrances.

The nucleic acid sequences which encode 9-hydroperoxide lyase may be used in various constructs, for example, as probes to obtain further sequences. Alternatively, these sequences may be used in conjunction with appropriate regulatory sequences to increase levels of the respective 9-HPO lyase of interest in a host cell for recovery or study of the enzyme *in vitro* or *in vivo* or to decrease levels of the respective 9-HPO lyase of interest for some applications when the host cell is a plant entity, including plant cells, plant parts (including but not limited to seeds, cuttings or tissues) and plants.

Thus, depending upon the intended use, the constructs may contain the nucleic acid sequence which encodes the entire 9-HPO lyase protein, or a portion thereof. For example, where antisense inhibition of a given 9-HPO lyase protein is desired, the entire 9-HPO lyase sequence is not required. Furthermore, where 9-HPO lyase constructs are intended for use as probes, it may be advantageous to prepare constructs containing only a particular portion of a 9-HPO lyase encoding sequence, for example a sequence which is discovered to encode a highly conserved 9-HPO lyase region.

As discussed above, nucleic acid sequence encoding a plant or other 9-HPO lyase of this invention may include genomic, cDNA or mRNA sequence. By "encoding" is meant that the sequence corresponds to a particular amino acid sequence either in a sense or anti-sense orientation. By "extrachromosomal" is meant that the sequence is outside of the plant genome of which it is naturally associated. By "recombinant" is meant that the sequence contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like.

A cDNA sequence may or may not contain pre-processing sequences, such as transit peptide sequences or targeting sequences to facilitate delivery of the 9-HPO lyase protein to a given organelle or membrane location. The use of any such precursor 9-HPO lyase DNA sequences is preferred for uses

in plant cell expression. A genomic 9-HPO lyase sequence may contain the transcription and translation initiation regions, introns, and/or transcript termination regions of the plant 9-HPO lyase, which sequences may be used in a variety of DNA constructs, with or without the 9-HPO lyase structural gene. Thus, nucleic acid sequences corresponding to the 9-HPO lyase of this invention may also provide signal sequences useful to direct protein delivery into a particular organellar or membrane location, 5' upstream non-coding regulatory regions (promoters) having useful tissue and timing profiles, 3' downstream non-coding regulatory regions useful as transcriptional and translational regulatory regions, and may lend insight into other features of the gene.

Once the desired plant or other 9-HPO lyase nucleic acid sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized. In the structural gene, one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

The nucleic acid or amino acid sequences encoding a plant or other 9-HPO lyase of this invention may be combined with other non-native, or "heterologous", sequences in a variety of ways. By "heterologous" sequences is meant any sequence which is not naturally found joined to the native (or wild-type) 9-HPO lyase, including, for example, combinations of nucleic acid sequences from the same plant which are not naturally found joined together.

The DNA sequence encoding a plant or other 9-HPO lyase of this invention may be employed in conjunction with all or part

of the gene sequences normally associated with the 9-HPO lyase. In its component parts, a DNA sequence encoding 9-HPO lyase is combined in a DNA construct having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription and translation in a host cell, the DNA sequence encoding plant 9-HPO lyase and a transcription and translation termination region.

Potential host cells include both prokaryotic and eukaryotic cells. A host cell may be unicellular or found in a multicellar differentiated or undifferentiated organism depending upon the intended use. Cells of this invention may be distinguished by having a 9-HPO lyase foreign to the wild-type cell present therein, for example, by having a recombinant nucleic acid construct encoding a 9-HPO lyase therein not native to the host species.

Prokaryotic cells include gram negative as well as gram positive bacteria, for example E. coli, and B. subtilis strains. Suitable examples are well known to the skilled artisan. As described in more detail in the examples that follow, an HPO lyase isolated from cucumber hypocotyl is expressed in E coli, strain M15. The protein expressed from the E coli is capable of producing the aldehyde 3(Z)-nonenal and 2(E)-nonenal from linoleic acid 9-hydroperoxide. Thus, the HPO lyase isolated from the cucumber hypocotyl encodes a 9-HPO lyase.

Eukaryotic host cells include fungi, including yeasts, insect cells, and plant cells. Methods for the expression of DNA sequences of interest in yeast cells are known in the art and are generally described in "Guide to yeast genetics and molecular biology", Guthrie and Fink, eds. Methods in enzymology , Academic Press, Inc. Vol 194 (1991) and Gene expression technology", Goeddel ed, Methods in Enzymology, Academic Press, Inc., Vol 185 (1991). In addition, methods for the expression of 13-HPO lyase genes are described in European patent Application EP 0 801 133 A2, the entirety of which is incorporated herein by reference.

The fungal recombinant vector may be any vector which can be conveniently subjected to recombinant DNA procedures. The

choice of a vector will typically depend on the compatibility of the vector with the fungal host cell into which the vector is to be introduced. The vector may be a linear or a closed circular plasmid. The vector system may be a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the fungal host.

The fungal vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the fungal cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. For integration, the vector may rely on the nucleic acid sequence of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the fungal host. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, there should be preferably two nucleic acid sequences which individually contain a sufficient number of nucleic acids, preferably 400bp to 1500bp, more preferably 800bp to 1000bp, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. These nucleic acid sequences may be any sequence that is homologous with a target sequence in the genome of the fungal host cell and, furthermore, may be non-encoding or encoding sequences.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of origin of replications for use in a yeast host cell are the

2 micron origin of replication and the combination of CEN3 and ARS 1. Any origin of replication may be used which is compatible with the fungal host cell of choice.

The fungal vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides, for example biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs and the like. The selectable marker may be selected from the group including, but not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), hygB (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase) and *sC* (sulfate adenyltransferase) and *trpC* (anthranilate synthase). Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* markers of *Aspergillus nidulans* or *Aspergillus oryzae* and the bar marker of *Streptomyces hygroscopicus*. Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/17243, the entirety of which is herein incorporated by reference. A nucleic acid sequence of the present invention may be operably linked to a suitable promoter sequence. The promoter sequence is a nucleic acid sequence which is recognized by the fungal host cell for expression of the nucleic acid sequence. The promoter sequence contains transcription and translation control sequences which mediate the expression of the protein or fragment thereof.

A promoter may be any nucleic acid sequence which shows transcriptional activity in the fungal host cell of choice and may be obtained from genes encoding polypeptides either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of a nucleic acid construct of the invention in a filamentous fungal host are promoters obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger*

or *Aspergillus awamori* glucoamylase (glaA), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase and hybrids thereof. In a yeast host, a useful promoter is the *Saccharomyces cerevisiae* enolase (eno-1) promoter. Particularly preferred promoters are the TAKA amylase, NA2-tpi (a hybrid of the promoters from the genes encoding *Aspergillus niger* neutral alpha -amylase and *Aspergillus oryzae* triose phosphate isomerase) and glaA promoters.

A protein or fragment thereof encoding nucleic acid molecule of the present invention may also be operably linked to a terminator sequence at its 3' terminus. The terminator sequence may be native to the nucleic acid sequence encoding the protein or fragment thereof or may be obtained from foreign sources. Any terminator which is functional in the fungal host cell of choice may be used in the present invention, but particularly preferred terminators are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase and *Saccharomyces cerevisiae* enolase.

A protein or fragment thereof encoding nucleic acid molecule of the present invention may also be operably linked to a suitable leader sequence. A leader sequence is a nontranslated region of a mRNA which is important for translation by the fungal host. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the protein or fragment thereof. The leader sequence may be native to the nucleic acid sequence encoding the protein or fragment thereof or may be obtained from foreign sources. Any leader sequence which is functional in the fungal host cell of choice may be used in the present invention, but particularly preferred leaders are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase and *Aspergillus oryzae* triose phosphate isomerase.

A polyadenylation sequence may also be operably linked to the 3' terminus of the nucleic acid sequence of the present

invention. The polyadenylation sequence is a sequence which when transcribed is recognized by the fungal host to add polyadenosine residues to transcribed mRNA. The polyadenylation sequence may be native to the nucleic acid sequence encoding the protein or fragment thereof or may be obtained from foreign sources. Any polyadenylation sequence which is functional in the fungal host of choice may be used in the present invention, but particularly preferred polyadenylation sequences are obtained from the genes encoding *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase and *Aspergillus niger* alpha-glucosidase.

To avoid the necessity of disrupting the cell to obtain the protein or fragment thereof and to minimize the amount of possible degradation of the expressed protein or fragment thereof within the cell, it is preferred that expression of the protein or fragment thereof gives rise to a product secreted outside the cell. To this end, a protein or fragment thereof of the present invention may be linked to a signal peptide linked to the amino terminus of the protein or fragment thereof. A signal peptide is an amino acid sequence which permits the secretion of the protein or fragment thereof from the fungal host into the culture medium. The signal peptide may be native to the protein or fragment thereof of the invention or may be obtained from foreign sources. The 5' end of the coding sequence of the nucleic acid sequence of the present invention may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted protein or fragment thereof. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted protein or fragment thereof. The foreign signal peptide may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide may simply replace the natural signal peptide to obtain enhanced secretion of the desired protein or fragment thereof. The

foreign signal peptide coding region may be obtained from a glucoamylase or an amylase gene from an *Aspergillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the alpha-factor from *Saccharomyces cerevisiae*, or the calf preprochymosin gene. An effective signal peptide for fungal host cells is the *Aspergillus oryzae* TAKA amylase signal, *Aspergillus niger* neutral amylase signal, the *Rhizomucor miehei* aspartic proteinase signal, the *Humicola lanuginosus* cellulase signal, or the *Rhizomucor miehei* lipase signal. However, any signal peptide capable of permitting secretion of the protein or fragment thereof in a fungal host of choice may be used in the present invention.

A protein or fragment thereof encoding nucleic acid molecule of the present invention may also be linked to a propeptide coding region. A propeptide is an amino acid sequence found at the amino terminus of aproprotein or proenzyme. Cleavage of the propeptide from the proprotein yields a mature biochemically active protein. The resulting polypeptide is known as a propolypeptide or proenzyme (or a zymogen in some cases). Propolypeptides are generally inactive and can be converted to mature active polypeptides by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide or proenzyme. The propeptide coding region may be native to the protein or fragment thereof or may be obtained from foreign sources. The foreign propeptide coding region may be obtained from the *Saccharomyces cerevisiae* alpha-factor gene or *Myceliophthora thermophila* laccase gene (WO 95/33836, the entirety of which is herein incorporated by reference).

The procedures used to ligate the elements described above to construct the recombinant expression vector of the present invention are well known to one skilled in the art (see, for example, Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd ed., Cold Spring Harbor, N.Y., (1989)).

The present invention also relates to recombinant fungal host cells produced by the methods of the present invention which are advantageously used with the recombinant vector of the present invention. The cell is preferably transformed

with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome. The choice of fungal host cells will to a large extent depend upon the gene encoding the protein or fragment thereof and its source. The fungal host cell may, for example, be a yeast cell or a filamentous fungal cell.

"Yeast" as used herein includes *Ascosporogenous* yeast (*Endomycetales*), *Basidiosporogenous* yeast and yeast belonging to the *Fungi Imperfecti* (*Blastomycetes*). The *Ascosporogenous* yeasts are divided into the families *Spermophthoraceae* and *Saccharomycetaceae*. The latter is comprised of four subfamilies, *Schizosaccharomycoideae* (for example, genus *Schizosaccharomyces*), *Nadsonioideae*, *Lipomycoideae* and *Saccharomycoideae* (for example, genera *Pichia*, *Kluyveromyces* and *Saccharomyces*). The *Basidiosporogenous* yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium* and *Filobasidiella*. Yeast belonging to the *Fungi Imperfecti* are divided into two families, *Sporobolomycetaceae* (for example, genera *Sorobolomyces* and *Bullera*) and *Cryptococcaceae* (for example, genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner *et al.*, *Soc. App. Bacteriol. Symposium Series* No. 9, (1980), the entirety of which is herein incorporated by reference). The biology of yeast and manipulation of yeast genetics are well known in the art (*see*, for example, *Biochemistry and Genetics of Yeast*, Bacil *et al.* (ed.), 2nd edition, 1987; *The Yeasts*, Rose and Harrison (eds.), 2nd ed., (1987); and *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.* (eds.), (1981), all of which are herein incorporated by reference in their entirety).

The recombinant fungal host cells of the present invention may further comprise one or more sequences which encode one or more factors that are advantageous in the expression of the protein or fragment thereof, for example, an activator (e.g., a trans-acting factor), a chaperone and a processing protease. The nucleic acids encoding one or more

of these factors are preferably not operably linked to the nucleic acid encoding the protein or fragment thereof. An activator is a protein which activates transcription of a nucleic acid sequence encoding a polypeptide (Kudla *et al.*, *EMBO* 9:1355-1364(1990); Jarai and Buxton, *Current Genetics* 26:2238-244(1994); Verdier, *Yeast 6*:271-297(1990), all of which are herein incorporated by reference in their entirety). The nucleic acid sequence encoding an activator may be obtained from the genes encoding *Saccharomyces cerevisiae* heme activator protein 1 (hap1), *Saccharomyces cerevisiae* galactose metabolizing protein 4 (gal4) and *Aspergillus nidulans* ammonia regulation protein (areA). For further examples, see Verdier, *Yeast 6*:271-297 (1990); MacKenzie *et al.*, *Journal of Gen. Microbiol. 139*:2295-2307 (1993), both of which are herein incorporated by reference in their entirety). A chaperone is a protein which assists another protein in folding properly (Hartl *et al.*, *TIBS 19*:20-25 (1994); Bergeron *et al.*, *TIBS* 19:124-128 (1994); Demolder *et al.*, *J. Biotechnology* 32:179-189 (1994); Craig, *Science 260*:1902-1903(1993); Gething and Sambrook, *Nature* 355:33-45 (1992); Puig and Gilbert, *J Biol. Chem. 269*:7764-7771 (1994); Wang and Tsou, *FASEB Journal* 7:1515-11157 (1993); Robinson *et al.*, *Bio/Technology 1*:381-384 (1994), all of which are herein incorporated by reference in their entirety). The nucleic acid sequence encoding a chaperone may be obtained from the genes encoding *Aspergillus oryzae* protein disulphide isomerase, *Saccharomyces cerevisiae* calnexin, *Saccharomyces cerevisiae* BiP/GRP78 and *Saccharomyces cerevisiae* Hsp70. For further examples, *see* Gething and Sambrook, *Nature 355*:33-45 (1992); Hartl *et al.*, *TIBS 19*:20-25 (1994). A processing protease is a protease that cleaves a propeptide to generate a mature biochemically active polypeptide (Enderlin and Ogrydziak, *Yeast 10*:67-79 (1994); Fuller *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 86*:1434-1438 (1989); Julius *et al.*, *Cell 37*:1075-1089 (1984); Julius *et al.*, *Cell 32*:839-852 (1983), all of which are incorporated by reference in their entirety). The nucleic acid sequence encoding a processing protease may be obtained from the genes encoding *Aspergillus niger* Kex2, *Saccharomyces cerevisiae*

dipeptidylaminopeptidase, *Saccharomyces cerevisiae* Kex2 and *Yarrowia lipolytica* dibasic processing endoprotease (xpr6). Any factor that is functional in the fungal host cell of choice may be used in the present invention.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 81*:1470-1474 (1984), both of which are herein incorporated by reference in their entirety. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, *Gene 78*:147-156 (1989), the entirety of which is herein incorporated by reference. Yeast may be transformed using the procedures described by Becker and Guarente, In: Abelson and Simon, (eds.), *Guide to Yeast Genetics and Molecular Biology, Methods Enzymol.* Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, *J. Bacteriology 153*:163 (1983); Hinnen *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.) 75*:1920 (1978), all of which are herein incorporated by reference in their entirety.

The present invention also relates to methods of producing the protein or fragment thereof comprising culturing the recombinant fungal host cells under conditions conducive for expression of the protein or fragment thereof. The fungal cells of the present invention are cultivated in a nutrient medium suitable for production of the protein or fragment thereof using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the protein or fragment thereof to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (*see, e.g.*, Bennett and LaSure (eds.), *More Gene Manipulations in Fungi*, Academic Press, CA, (1991), the entirety of which is

herein incorporated by reference). Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection, Manassas, VA). If the protein or fragment thereof is secreted into the nutrient medium, a protein or fragment thereof can be recovered directly from the medium. If the protein or fragment thereof is not secreted, it is recovered from cell lysates.

The expressed protein or fragment thereof may be detected using methods known in the art that are specific for the particular protein or fragment. These detection methods may include the use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, if the protein or fragment thereof has enzymatic activity, an enzyme assay may be used. Alternatively, if polyclonal or monoclonal antibodies specific to the protein or fragment thereof are available, immunoassays may be employed using the antibodies to the protein or fragment thereof. The techniques of enzyme assay and immunoassay are well known to those skilled in the art.

The resulting protein or fragment thereof may be recovered by methods known in the arts. For example, the protein or fragment thereof may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The recovered protein or fragment thereof may then be further purified by a variety of chromatographic procedures, e.g., ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like.

Methods for the expression of DNA sequences of interest in insect host cells are also well known in the art, and are reviewed by Lucow and Summers, (1988) *Bio/technology* 6:47-55, the entirety of which is incorporated herein by reference.

In a preferred embodiment, plant host cells are employed in transformation experiments with constructs containing DNA sequences coding 9-HPO lyase enzymes expressed from constitutive or tissue enhanced promoters.

Depending upon the host, the regulatory regions will vary, including regions from viral, plasmid or chromosomal genes, or the like. For expression in prokaryotic or eukaryotic microorganisms, particularly unicellular hosts, a wide variety of constitutive or regulatable promoters may be employed. Expression in a microorganism can provide a ready source of the plant enzyme. Among transcriptional initiation regions which have been described are regions from bacterial and yeast hosts, such as *E. coli, B. subtilis, Sacchromyces cerevisiae,* including genes such as beta-galactosidase, T7 polymerase, tryptophan E and the like.

In a preferred embodiment, the constructs will involve regulatory regions functional in plants which provide for modified production of 9-HPO lyase, and, possibly, modification of the fatty acid composition. The open reading frame coding for the plant HPO lyase or functional fragment thereof will be joined at its 5′ end to a transcription initiation regulatory region. In embodiments wherein the expression of the 9-HPO lyase protein is desired in a plant host, the use of all or part of the complete plant HPO lyase gene is desired; namely all or part of the 5′ upstream non-coding regions (promoter) together with the structural gene sequence and 3′ downstream non-coding regions may be employed.

If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having transcription initiation regions derived from one gene source and translation initiation regions derived from a different gene source, numerous transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the structural gene functions. The transcription/translation initiation regions corresponding to such structural genes are found immediately 5′ upstream to the respective start codons. Among transcriptional initiation regions used for plants are such regions associated with the T-DNA structural genes such as for nopaline and mannopine synthases, the 19S and 35S promoters from CaMV, and the 5′ upstream regions from other plant genes such as napin, ACP, SSU, PG, zein, phaseolin E,

and the like. Enhanced promoters, such as double 35S, are also available for expression of HPO lyase sequences. For such applications when 5' upstream non-coding regions are obtained from other genes regulated during seed maturation, those preferentially expressed in plant embryo tissue, such as ACP and napin-derived transcription initiation control regions, are desired. Such "seed-specific promoters" may be obtained and used in accordance with the teachings of issued U.S. Patent Numbers 5,608,152 and 5,530,194, which references are hereby incorporated by reference. Transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts, are considered desirable for expression in order to minimize any disruptive or adverse effects of the gene product.

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence encoding the plant HPO lyase or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. Where the transcript termination region is from a different gene source, it will contain at least about 0.25 kb, preferably about 1-3 kb of sequence 3' to the structural gene from which the termination region is derived.

Plant expression or transcription constructs having a 9-HPO lyase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of tissues for edible uses. Most especially preferred are seed, fruit, vegetable and leaf crops. Plants of interest include, but are not limited to, *Brassica* species, soybean, corn, tomato, strawberry, bell pepper and melon. Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledenous and monocotyledenous species alike and will be

readily applicable to new and/or improved transformation and regulation techniques.

In order to increase lipid peroxidation, and thereby increasing "melon" flavors/fragrances, in a plant tissue, coexpression of a plant or other 9-HPO lyase in a plant tissue with a second gene involved in lipid peroxidation may also find use in the present invention. For example, coexpression of a 9-HPO lyase sequence in a plant tissue with a DNA sequence encoding for another protein involved in lipid peroxidation, such as a lipoxygenase may increase lipid peroxidation and increase the total short-chain aldehydes produced in the plant tissue. Such an increase in short-chain aldehydes may increase the "melon" flavor in an edible plant tissue.

The method of transformation in obtaining such transgenic plants is not critical to the instant invention, and various methods of plant transformation are currently available. Furthermore, as newer methods become available to transform crops, they may also be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation. In addition, techniques of microinjection, DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host,

viral immunity or the like. Depending upon the number of different host species the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall formation), the latter being permissible, so long as the *vir* genes are present in the transformed *Agrobacterium* host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming host plant cells, the expression or transcription construct bordered by the T-DNA border region(s) will be inserted into a broad host range vector capable of replication in *E. coli* and *Agrobacterium*, there being broad host range vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta, *et al.*, (*Proc. Nat. Acad. Sci., U.S.A.* (1980) 77:7347-7351) and EPA 0 120 515, which are incorporated herein by reference. Alternatively, one may insert the sequences to be expressed in plant cells into a vector containing separate replication sequences, one of which stabilizes the vector in *E. coli*, and the other in *Agrobacterium*. See, for example, McBride and Summerfelt (*Plant Mol. Biol.* (1990) *14*:269-276), wherein the pRiHRI (Jouanin, *et al., Mol. Gen. Genet.* (1985) 201:370-374) origin of replication is utilized and provides for added stability of the plant expression vectors in host *Agrobacterium* cells.

Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed Agrobacterium and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, kanamycin, the

aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be grown to seed and the seed used to establish repetitive generations and for isolation of vegetable oils.

Once transgenic hosts have been obtained which express the 9-HPO lyase encoding sequence, a number of methods are available in the art for the analysis of the expression and protein activity. Methods for the analysis of 9-HPO lyase expression, such as Southern and Northern hybridizations and Western immuno blot assays are generally described in Maniatis, *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. Methods for the analysis of HPO lyase activity are described in Matsui, *et al.* (1996) *FEBS Letters* 394:21-24; Rehbock, *et al.* (1998) *Fresenius J. Analytical Chem* 360:830-832; Nunez, *et al.* (1998) *Lipids* 33(5):533-538; and in European Patent Application EP 0 801 133 A2.

Host plants expressing the 9-HPO lyase sequences of the present invention find use in the production of volatile aldehydes referred to as melon note compounds. Such compounds can be used to sharpen and enhance flavored products, such as those having fruit flavors. Such compounds and uses are described by Morris, (1981) *Perfumer & Flavorist* vol 6 no1, and Clark, (1990) *Perfumer and Flavorist* vol 15, the entireties of which are incorporated herein by reference.

Host cells expressing the 9-HPO lyases of the present invention provide a novel source of volatile aldehydes contributing to "melon" notes for use in various applications. Furthermore, the host cells may also contain constructs providing for a increased production of enzymes involved in lipid peroxidation, for example lipoxygenase. In addition, the host cells may also produce an increased amount of a particular fatty acid, or have a general increase in fatty acids. Such host cells may be obtained using traditional breeding techniques, including mutagenesis, as well as hosts genetically engineered with such an altered fatty acid composition.

Furthermore, plant host cells containing a construct providing for the expression of the 9-HPO lyase sequences of the present invention find use as a source for aldehydes in reactions for the production of alcohols for use in flavorings and aromatic products. Such methods are known in the art and are described for example in U.S. Patent Number 5,695,973 and in PCT Publication WO 95/26413 the entireties of which are incorporated herein by reference. Generally, a mixture of aldehydes and alcohols are obtained from such methods. The methods generally involve a reaction mixture containing at least one unsaturated fatty acid, a plant material having a relatively high amount of enzyme activity of lipoxygenase and hydroperoxide lyase, and a source of alcohol dehydrogenase.

The unsaturated fatty acid may vary and include a single unsaturated fatty acid species as well as mixtures of several unsaturated fatty acids. The fatty acids are provided in a free acid form, and examples include, but are not limited to oleic acid, linoleic acid, linolenic acid (alpha and gamma forms), arachidonic acid, eicosapentaenoic acid, and ricinoleic acid.

Sources of the alcohol dehydrogenase include yeasts, as well as non-yeast molds. The alcohol dehydrogenase has the ability to convert an aldehyde to an alcohol. The yeast and non-yeast molds further provide a source of nicotine adenine dinucleotide (NADH) as a reducing agent.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

Example 1 Identification of Arabidopsis HPO Lyase Sequences

A nucleic acid fragment encoding hydroperoxide lyase from Bell pepper has been previously cloned and sequenced (Matsui, *et al.* (1996) *supra*). The nucleotide sequence was used to search Genbank for HPO lyase related sequences. One accession identified from Genbank (Accession Number Z97339, (http://www.ncbi.nlm.nih.gov/web/Genbank/Index.html)) containing a genomic sequence from *Arabidopsis* was reported to encode an allene oxide synthase.

Sequence comparisons between the bell pepper HPO lyase, *Arabidopsis* allene oxide synthase (Laudert, *et al.* (1996) *supra*) and the *Arabidopsis* HPO lyase-like sequence from Genbank using Genetyx Mac (Software Development Co. Ltd.) indicated that the *Arabidopsis* HPO lyase-like sequence is more similar to the bell pepper HPO lyase (57% identity) (see Figure 2) than to the allene oxide synthase sequence (39% identity) (see Figure 3).

Example 2 Construction of *Arabidopsis* cDNA libraries

Total RNA from seedling, inflorescence, and silique tissues of *Arabidopsis thaliana* is isolated for use in construction of complementary (cDNA) libraries. The procedure is an adaptation of the DNA isolation protocol of Webb and Knapp (D.M. Webb and S.J. Knapp, (1990) Plant Molec. Reporter, 8, 180-185). The following description assumes the use of 1g fresh weight of tissue. Frozen seed tissue is powdered by grinding under liquid nitrogen. The powder is added to 10ml REC buffer (50mM Tris-HCl, pH 9, 0.8M NaCl, 10mM EDTA, 0.5% w/v CTAB (cetyltrimethyl-ammonium bromide)) along with 0.2g insoluble polyvinylpolypyrrolidone, and ground at room temperature. The homogenate is centrifuged for 5 minutes at 12,000 x*g* to pellet insoluble material. The resulting

supernatant fraction is extracted with chloroform, and the top phase is recovered.

The RNA is then precipitated by addition of 1 volume RecP (50mM Tris-HCL pH9, 10mM EDTA and 0.5% (w/v) CTAB) and collected by brief centrifugation as before. The RNA pellet is redissolved in 0.4 ml of 1M NaCl. The RNA pellet is redissolved in water and extracted with phenol/chloroform. Sufficient 3M potassium acetate (pH 5) is added to make the mixture 0.3M in acetate, followed by addition of two volumes of ethanol to precipitate the RNA. After washing with ethanol, this final RNA precipitate is dissolved in water and stored frozen.

Alternatively, total RNAs may be obtained using TRIzol reagent (BRL Life Technologies, Gaithersburg, MD) following the manufacturers protocol.

Complementary DNAs (cDNA) are obtained from the RNAs using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) following the manufacturers directions.

Example 3 Cloning of HPO Lyase Sequences

In order to characterize the protein encoded by the *Arabidopsis* cDNA GenBank sequence, the entire coding region corresponding to the *Arabidopsis* HPO lyase-like cDNA was obtained. (Figure 1) Synthetic oligo-nucleotide primers are designed to amplify the 5' and 3' ends from the HPO lyase-like sequence from RNA obtained in Example 2. Primers are designed according to the *Arabidopsis* HPO lyase-like sequence and are used in Rapid Amplification of cDNA Ends (RACE) reactions (Frohman *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:8998-9002). Amplification of flanking sequences from cDNA clones are performed using the Marathon cDNA Amplification kit (Clontech) according to the manufacturers protocol.

A pair of primers were designed to amplify the 5' and 3' regions from the *Arabidopsis* HPO lyase-like cDNA from the libraries described in example 2 above. These two primers, HPOL28 (for 3' RACE, 5'-CGGTTCCTCTGCGCCTCTCTCGCCGGCG-3') and HPOL21 (for 5' RACE, 5'-GCGGAACCGGAGGACTAAAACGCAGC-3') are used in PCR reactions with Adapter specific primers (AP1 5'-

CCATCCTAATACGACTCACTATAGGGC-3') provided in the Marathon cDNA Amplification Kit. For amplification of the 5' region of the HPO lyase-like cDNA the primers AP1 and HPOL 21 were used, and for the amplification of the 3' region the AP1 primer was used in a reaction with the primer HPOL28. The cycle conditions used are: 94°C for 1 minute followed by 5 rounds of 94°C for 5 seconds, 72°C for 4 minutes, followed by 5 rounds of amplification using 94°C for 5 seconds, 70°C for 4 minutes, and finally 25 cycles of 94°C for 5 seconds, and 68°C for 4 minutes.

A single fragment of 1100 bp was obtained from the 3'RACE reaction with RNA obtained from the silique tissue described above. To confirm that the PCR product contained sequence corresponding to the HPO lyase-like sequence, a second round of PCR reactions using the same conditions described above was performed with the gel purified 1100 bp fragment. A reaction was performed with the primers HPOL13 (5'-CTTGGCGTAGTTCCTCAGCCTCTTG-3') and AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3') to amplify an approximately 1000 bp fragment as a confirmation of the HPO lyase-like sequence. The reamplified 1000 bp fragment was gel purified and cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) to create the plasmid pCGN8094.

The 5' RACE reaction produced many non-specific fragments. A 1000 bp fragment was excised from the gel and cloned into the pCR2.1 TOPO(Invitrogen) cloning vector to create the plasmid pCGN8091.

Figure 1 discloses the complete nucleotide sequence of the *Arabidopsis* HPO lyase.

Example 4 Preparation HPO Lyase Expression Constructs

A set of constructs are prepared for transformation into either plant or bacterial hosts to further characterize the *Arabidopsis* HPO lyase-like sequence. The 5' RACE product in pCGN8091 was PCR amplified using the primers Alex2 (5'-CGGGATCCATGTTGTTGAGAACGATGGCGGCG-3') and Alex4 (5'-CAATCTCCGGCGTTCTCGTCG-3'). The Alex2 primer contains the

restriction endonuclease site *Bam*HI for the convenient cloning of the PCR product into the pQE30 expression vector (Qiagen, Hilden, Germany) in frame with the ATG start codon of the vector. In addition to the oligonucleotide primers (0.2μM each), the PCR reaction mix contained 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.0% glycerol, 0.2 mM Tris-HCl (pH 8.3), 4.6 mM KCl, 1.5 mM EDTA, 15 μM dithiothreitol, 7.3 μgm/ml BSA, 1.1 mM KOAc and 0.1 units *Pfu* DNA polymerase (BRL Life Technologies, Gaithersburg, MD). The mixtures were amplified using the following conditions: 1 cycle of 95°C for 10 minutes; 30 cycles of 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes; and, 1 cycle of 72°C for 7 minutes in a Perkin-Elmer 9800 thermocycler. The resulting PCR product was digested with *Bam*HI and *Hind*III and ligated into the vector pQE30 to create the vector pCGN8099. The 3' terminus of the *Arabidopsis* HPO lyase was cloned into the *Hind*III site of pCGN8099 from pCGN8094 to create the *E. coli* expression vector pCGN8100

A binary vector for plant transformation, pCGN5138, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as an *Hind*III/*Eco*RI fragment with a polylinker containing unique restriction endonuclease sites, *Hind*III, *Sse*I/*Pst*I, *Not*I, *Bam*HI, *Swa*I, *Xba*I, *Pac*I, *Asc*I, and *Asp*718.

An antisense construct of the *Arabidopsis* HPO lyase-like nucleotide sequence was prepared for transformation of *Arabidopsis*. The nucleic acid sequence encoding the 5' 1000 bp nucleotides from pCGN8091 were cloned as an *Eco*RI fragment into the plasmid pBluescript II SK (Stratagene, La Jolla, CA) to create the vector pCGN8093. The 3' RACE product from pCGN8090 was cloned as a *Hind*III fragment into pCGN8093 to create a full length HPO lyase coding sequence in the plasmid pCGN8094. The *Kpn*I site of pCGN8094 was removed by digesting with *Kpn*I and filling in the site with Klenow fragment, and the HPO lyase coding sequence was cloned from this plasmid as a *Sma*I fragment into the *Stu*I site of pCGN8059. This yields

the plasmid pCGN8101. The plasmid pCGN8059 contains a multiple cloning site downstream of the 35S promoter and the hsp70 leader sequence to allow for the cloning of sequences for expression from the 35S promoter sequence. This vector also contains the nopaline synthase transcription termination (nos 3') sequences (Fraley et al., *Proc. Natl. Acad. Sci* (1983) 80:4803-4807 and Depicker et al., *J. Molec. Appl. Genet.* (1982) 1: 562-573). The fragment containing the 35S promoter/hsp70 leader, antisense *Arabidopsis* HPO lyase sequence, and *nos*3' termination sequence was cloned from pCGN8101 as a *Not*I fragment into the same site of pCGN5138 to create the antisense expression construct pCGN8102.

Example 5 E coli Expression

The expression vector pCGN8100 was transformed into *E coli* (strain M15, Qiagen, Hilden, Germany) using a calcium chloride procedure described in Maniatis, *et al.* ((1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Transformed colonies were screened by Western immunoblot analysis for expression of the HPO Lyase protein using antibodies raised to the bell pepper HPO lyase as described in Shibata, *et al.* (1995) *Plant Cell Physiol.* 97:1059-1072.

Hydroperoxide lyase activity was determined by gas chromotography (GC) methods described by Matsui, *et al.* (1991), *Phytochemistry*, 30:2109-2113 using linolenic acid 13-hydroperoxide as a substrate.

TABLE 1

Sample	Area	nmole	nmole/10min/mg
8100	24677	130	153
Control	4089	28	24

The results of the GC analysis shown in Table 1, confirms that the *Arabidopsis* HPO lyase-like sequence encodes a 13-HPO lyase enzyme.

Example 6 Identification of Additional HPO lyase Sequences

Additional HPO lyase-like sequences are obtained from tomato (*Lycopersicon esculentum* Mill. Cv UC82B) green immature fruit (ca 20 mm) and cucumber (*Cucumis sativus* L. cv Suyo) hypocotyl (of 3 day old seedlings) tissues. Total RNA was isolated using TRIzol reagent (Gibco-BRL Life Technologies, Gaithersburg, MD) following the manufacturers protocol.

Complementary DNAs (cDNA) are obtained using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) following the manufacturers directions.

The sequences of HPO lyase from bell pepper (Matsui, *et al.* (1996) *supra*), banana (European Patent Application, Publication Number EP 0 801 133 A2) and *Arabidopsis* were aligned using ClustalW (http://www.clustalw.genome.ac.jp/) and seven conserved peptide sequences were identified (see Figure 7 for positions, Table 2 for a listing).

Table 2

	Peptide Sequence	Primer Name	Oligonucleotide Sequence
1	PGSYG	HPOL1S	5'-ATNCCNGGNWSNTAYGG-3'
2	QPLEEI	HPOL2S	5'-CARCCNYTNGARGARAT-3'
		HPOL2AS	5'-ATYTCYTCNARNGGYTG-3'
3	GFNAYGG	HPOL3S	5'- GGNTTYAAYGCNTWYGGNGG-3'
		HPOL3AS	5'-CCNCCRSANGCRTTRAANCC-3'
4	YQPLVM	HPOL4S	5'-TAYCARCCNYTNGTNATG-3'
		HPOL4AS	5'-CATNACNARNGGYTGRTA-3'
5	VFDEPE	HPOL5S	5'-GTNTTYGAYGANCCNGA-3'
		HPOL5AS	5'-TCNGGNTCRTCRAANAC-3'
6	NGPQTG	HPOL6AS	5'-CCNGTYTWNGGNCCRTT-3'
7	NKQCAAKD	HPOL7AS	5'-CYTTNGCNGCRCAYTGYTTRTT-3'

A set of synthetic oligonucleotides (Table 2) are synthesized for use in polymerase chain reactions with the cDNAs obtained above to identify sequences which are homologous to HPO lyase sequences. The PCR reactions are carried out using Advantage cDNA Polymerase Mix (Clonetech, Palo Alto,CA) using the reaction conditions according to the manufacturers protocol. The letter "S" in the oligonucleotide name designates a PCR primer designed to amplify the sense strand, or forward reaction primer. The letters "AS"

designates a PCR primer designed to amplify the antisense strand, or reverse reaction primer. In the oligonucleotide sequence, the letters "N" represents an A, C, G, or a T, the letter "S" represents a C or a G in that position, the letter "Y" represents a C or a T, and the letter "R" represents an A or a G in that position.

A single PCR product, of approximately 475 bp, was amplifed in reactions containing the primers 4HPOL3S and 11HPOL7AS, with the cDNAs obtained from both cucumber and tomato (described above). The 475 bp PCR product from tomato and cucumber were cloned into the plasmid pCR2.1TOPO (invitrogen) to yield the plasmids T15 (pCGN8305) and C15 (pCGN8309) respectively. In PCR reactions with 6HPOL4S and 11HPOL7AS, a single product, of approximately 200 bp, was obtained from amplification reactions with cDNA obtained from cucumber hypocotyl tissue. The 200 bp product was cloned into pCR2.1 TOPO (Invitrogen), to create the plasmid C17 (pCGN8308).

The nucleotide sequence of each PCR product was determined by automated sequencing. The sequences obtained are compared to nucleic acid and amino acid sequences of HPO lyase sequences from bell pepper, *Arabidopsis*, and banana leaf, as well as to DNA and amino acid sequences coding for allene oxide synthases from guayule ((1995) *J. Biol. Chem.* 270(15):8487-8494), flaxseed ((1993) *Proc Natl Acad Sci USA* 90(18):8519-8523) and *Arabidopsis*.

The results demonstrate that the T15 nucleic acid sequence is approximately 85% similar to the bell pepper HPO lyase DNA sequence and about 88% similar in the amino acid sequence. Furthermore, the T15 sequence is also at least about 55% similar to other HPO lyase nucleic acid sequences and at least about 57% similar in the amino acid sequence. In addition, the T15 amino acid sequence is only about 41% similar to the allene oxide synthase sequences. The C17 sequence also follows a similar pattern of similarity to the HPO lyase sequences. Thus, the T15 and C17 sequences encode proteins highly similar to HPO lyase.

However, the results of the sequence comparisons (Figure 8) demonstrate that the C15 nucleic acid sequence is between 50% and 54% similar to the other HPO lyase nucleic acid sequences and about 58% similar to the allene oxide synthase DNA sequences. Furthermore, the deduced amino acid sequence of C15 is between about 38% and 42% similar to the HPO lyase amino acid sequences and about 51% similar to the AOS amino acid sequences. Thus, the C15 sequence encodes a protein which is divergent from both the known HPO lyase sequences, and is more similar to allene oxide synthase sequences.

The T15, C15 and C17 sequences are used to search Genbank. Search results further confirm the sequences from T15 and C17 as being similar to HPO lyase sequences, while the sequence of C15 is more similar to allene oxide synthase sequences.

In order to obtain full length coding sequence for T15, C15 and C17, RACE PCR reactions are employed using the Marathon cDNA Amplification kit (Clontech) according to the manufacturers protocol, and the oligonucleotides shown in Table 3.

Table 3

1KMC10-1:	5'-CGGTGGAGATCCTCGCCACCGGTGCCGACCC-3'
2KMC10-2:	5'-CTTCCTTCACGGTTGTCCTCACTTCCTCCGCCAG-3'
3KMC17-1:	5'-TCCAGCAGCGCTGCCCCTTTCTCTCCCCGG-3'
4KMC17-2:	5'-CACTGTTTGTTCTTCTCGCTCGGTGTCCCCG-3'
5KMC10-3:	5'-GGGTCGGCACCGGTGGCGAGGATCTCCACCG-3'
6KMC10-4:	5'-CTGGCGGAGGAAGTGAGGACAACCGTGAAGGAAG-3'
7KMC17-3:	5'-CCGGGGAGAGAAAGGGGCAGCGCTGCTGG-3'
8KMC17-4:	5'-CGGGGACACCGAGCGAGAAGAACAAACAGTG-3'
9KMT15-1:	5'-GACTTGGTACTGGTGGACTAAGCCTAAGTGTTTC-3'
10KMT15-2:	5'-GGCTGATAACCACAAAGAAGCTCCCCTTTC-3'
11KMT15-3:	5'-GAAACACTTAGGCTTAGTCCACCAGTACCAAGTC-3'
12KMT15-4:	5'-GAAAGGGGAGCTTCTTTGTGGTTATCAGCC-3'

PCR products from the amplification reactions with DNA obtained from tomato and cucumber are cloned into pCR2.1 TOPO. The sequences of the 5' and 3'-RACE products from tomato

(pCGN8303 (5' RACE) and pCGN8304 (3' RACE)), cucumber, C15 (pCGN8302 (5'RACE) and pCGN8306 (3' RACE)) and C17 (pCGN8301 (5' RACE) and pCGN8307 (3' RACE)) are sequenced and aligned with the respective sequences obtained from pCGN8305, pCGN8309, and pCGN8308 to obtain preliminary full length sequences corresponding to a tomato HPO lyase-like sequence (Figure 4), a cucumber HPO lyase-like sequence (Figure 6) and a cucumber allene oxide synthase-like sequence (Figure 5).

Example 7 Preparation of Expression Constructs

A set of constructs are prepared for transformation into either plant or bacterial hosts to further characterize the novel sequence from cucumber. To create a full length coding sequence for the cucumber (C15) allene oxide synthase-like sequence, the sequences from the 5' RACE (pCGN8302) and 3' RACE (pCGN8306) were PCR amplified and combined at a unique restriction endonuclease site.

The 5' C15 sequence is amplified using primers (4KMC15ES1 5'- CGGGATCCATGGCTTCTTCCTCCCCTGAACTTC-3' and 5KMC15EAS2 5'-TGCCGACCCATTTCAGTATAGTGGG-3') in PCR amplification reactions described above. The primer 4KMC15EAS1 amplifies from the 5' region and contains the start codon (ATG), and a *Bam*HI site. The 3' C15 sequence is amplified using the AP1 primer provided in the Marathon Kit (BRL-Lifetechnologies, Gaithersburg, MD) and the primer 6KMC15ES3 (5'- TTCACACCATTCCCCTGCCTTTCTTCCC-3'). The sequence of the C15 full length clone is shown in Figure 6.

A. Bacterial Expression Construct

The 5' RACE PCR amplification product is digested with *Bam*HI and *Xba*I (unique site endogenous to the C15 sequence) and cloned into the expression vector pQE30 (Invitrogen) with the amplification product of the 3' RACE PCR reaction digested with *Xba*I and *Sma*I. This construct provides a full length encoding sequence of the C15 cDNA in the *E. coli* expression vector to create the vector pCGN8333. The full length sequence is also cloned into the plasmid pUC119 to create the vector pCGN8334.

B. Plant Expression Construct

A binary vector for plant transformation, pCGN5138, was constructed from pCGN1558 (McBride and Summerfelt, (1990) Plant Molecular Biology, 14:269-276). The polylinker of pCGN1558 was replaced as an *Hind*III/*Eco*RI fragment with a polylinker containing unique restriction endonuclease sites, *Hind*III, *Sse*I/*Pst*I, *Not*I, *Bam*HI, *Swa*I, *Xba*I, *Pac*I, *Asc*I, and *Asp*718.

The full length coding sequence of C15 is cloned to be expressed from the plant constitutive promoter 35S for expression in plants. The expression cassette is cloned into the binary vector pCGN5138 to create the vector pCGN8337.

Example 8 Expression of cucumber C15 in *E. coli*

The expression vector pCGN8333 was transformed into *E coli* (strain M15, Qiagen, Hilden, Germany) using a calcium chloride procedure described in Maniatis, *et al.* ((1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Transformed colonies were screened by Western immunoblot analysis for expression of the HPO Lyase protein using antibodies raised to the bell pepper HPO lyase as described in Shibata, *et al.* (1995) *Plant Cell Physiol.* 97:1059-1072.

Hydroperoxide lyase activity was determined by spectrophotometric and gas chromotography (GC) methods described by Matsui, *et al.* (1991), *Phytochemistry*, 30:2109-2113, using both linolenic acid 13-hydroperoxide and linolenic acid 9-hydroperoxide as substrates.

The results of the gas chromotography assay (Figure 9) demonstrate that the protein encoded by the cucumber C15 sequence has greater activity toward linolenic acid 9-hydroperoxide (Figure 9B) substrates than linolenic acid 13-hydroperoxide substrates (Figure 9A). The results of the spectrophotometric assays further demonstrate the preference of the protein encoded by cucumber HPO lyase nucleic acid sequence for 9-Hydroperoxide substrates. The results of the spectrophotometric assay are presented in Figure 10.

Thus, the cucumber C15 sequence represents the first known cloning of a nucleic acid sequence encoding a 9-hydroperoxide lyase.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claim.

Claims

What is Claimed is:

1. An isolated nucleic acid sequence coding a hydroperoxide lyase with activity toward fatty acid 9-hydroperoxides.

2. The sequence according to Claim 1, wherein said sequence has activity toward linolenic acid 9-hydroperoxide.

3. The sequence according to Claim 1, wherein said sequence is obtained from a plant source.

4. The sequence according to Claim 1, wherein said sequence is obtained from cucumber.

5. The sequence according to Claim 1, wherein said sequence is obtained from cucumber hypocotyl.

6. The sequence according to Claim 1, comprising the sequence shown in Figure 6.

7. A construct comprising a promoter functional in a host cell, a sequence encoding a 9-hydroperoxide lyase, and a transcriptional termination sequence.

8. A construct according to Claim 7 wherein said 9-hydroperoxide lyase sequence is isolated from a plant.

9. A construct according to Claim 7 wherein said 9-hydroperoxide lyase sequence is isolated from cucumber.

10. A method for increasing the resistance of a plant to a plant pathogen comprising expressing an HPO lyase from a construct according to Claim 7, wherein said HPO lysase encoding sequence is linked to DNA sequence capable of directing expression in a plant cell.

11. A method for increasing the volatile composition of a plant comprising expressing an HPO lyase from a construct according to Claim 7, wherein said HPO lysase encoding sequence is linked to DNA sequence capable of directing expression in a plant cell.

[GENETYX: Translation of Nucleotide into Amino Acids]

1997.11.19

Filename : Arabidopsis/lyase/cDNA/truncate
Sequence Size : 1385
Sequence Position: 1 - 1385

```
        10         20         30         40         50         60         70         80         90        100
ATGGCGGCGACTTCCCCGCGGCCACCACCGTCAACATCCCTAACATCTCAGCAGCCACCATCACCCCCCTCACAGCTTCCCCTCCGTACAATGCCGGGAT
M  A  A  T  S  P  R  P  P  P  S  T  S  L  T  S  Q  Q  P  P  S  P  P  S  Q  L  P  L  R  T  M  P  G  S

       110        120        130        140        150        160        170        180        190        200
CGTACGGCTGGCCGTTGGTTGGACCATTATCGGACCGTTTAGATTCCAAGGACCCGATAAGTTTTTCCGGACAAGAGCTGAGAAGTATAAGAGCACTGTG
  Y  G  W  P  L  V  G  P  L  S  D  R  L  D  S  K  D  P  I  S  F  S  G  Q  E  L  R  S  I  R  A  L  C

       210        220        230        240        250        260        270        280        290        300
TTCCGTACAAATATTCCTCCGACGTTTCCTTTCTTCGGCAACCCATCTTTTTGACATGGATCTAGTTGATAAAAGAGATGTTCTCATCGGAGACTTCCGG
 S  V  Q  I  F  L  R  R  F  L  S  S  A  T  H  L  F  D  M  D  L  V  D  K  R  D  V  L  I  G  D  F  R

       310        320        330        340        350        360        370        380        390        400
CCTAGCCTTGGGTTCTACGGCGGCGTTCGTGTTGGAGTTTATCTCGACACTACTGAGCCAAAGCACGCCAAGGTTCGTACATACGTTGCTTTGCTACTAT
P  S  L  G  F  Y  G  G  V  R  V  G  V  Y  L  D  T  T  E  P  K  H  A  K  V  R  T  Y  V  A  L  L  L  L

       410        420        430        440        450        460        470        480        490        500
TACATAAAAGGTTTCGCTATGGAAACACTAAAACGAAGCTCAAAAGTATGGCTACAAGAGCTTCGTTCAAACCTAAACATTTTCTGGGGAACAATCGAAT
  H  K  R  F  R  Y  G  N  T  K  T  K  L  K  S  M  A  T  R  A  S  F  K  P  K  H  F  L  G  N  N  R  I

       510        520        530        540        550        560        570        580        590        600
CCGAAATCTCCAAAAACGGTTCCTCTGCGCCTCTCTCGCCGGCGTTGACGCTTCCGTATCGCCGGACATCGCTGAGAACGGTTGGAAAACAATCAATACT
R  N  L  Q  K  R  F  L  C  A  S  L  A  G  V  D  A  S  V  S  P  D  I  A  E  N  G  W  K  T  I  N  T
                                                  HPCL 12

       610        620        630        640        650        660        670        680        690        700
TGGCTTGCGTTGCAAGTTATTCCCACTGCTAAACTTGGCGTAGTTCCTCAGCCTCTTGAAGAGATTTTACTTCATACTTGGCCTTATCCTTCTCTCTTAA
W  L  A  L  Q  V  I  P  T  A  K  L  G  V  V  P  Q  P  L  E  E  I  L  L  H  T  W  P  Y  P  S  L  L  I
                                                  HPCL 13
```

Fig. 1A

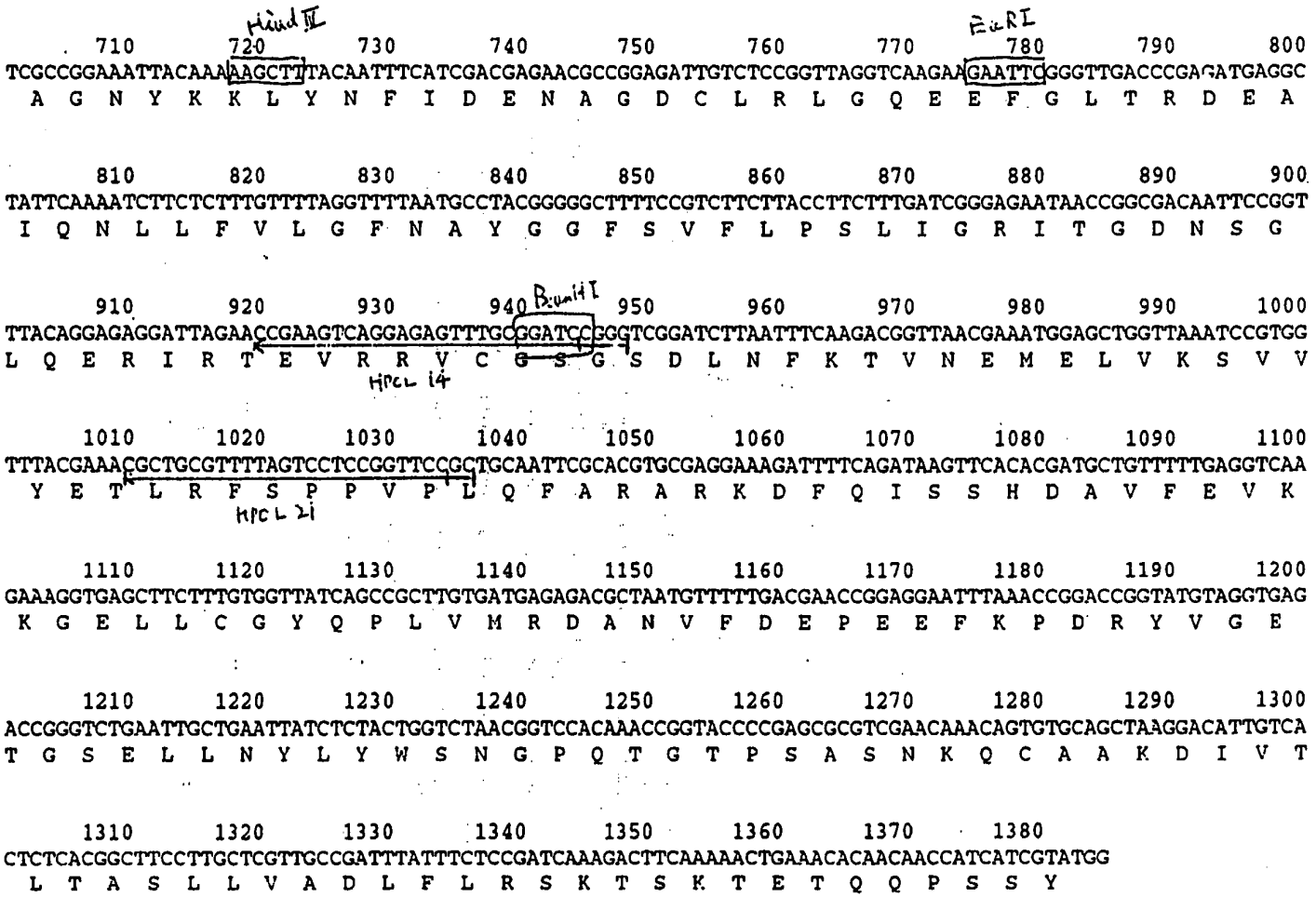

Fig. 1B

```
[ GENETYX : Amino Acid Sequence Homology Data ]

1st Amino Acid Sequence
  File Name          : Arabidopsis lyase AA
  Sequence Size      : 492

2nd Amino Acid Sequence
  File Name          : Bell pepper lyase AA
  Sequence Size      : 480

  Unit Size to compare = 2


[57.0% / 470 aa]

    1' MLLRTMAATSPRPPPSTSLTSQQPPSPPSQLPLRTMPGSYGWPLVGPLSDRLDYFWFQGP
    1*                  MIPIMSSAPLSTATPISLPVRKIPGSYGFPLLGPLWDRLDYNWFQKL

   61' DKFFRTRAEKYKSTVFRTNIPPTFPFFGNVNPNIVAVLDVKSFSHLFDMDLVDKRDVLIG
   48* PDFFSKRVEKYNSTVFRTNVPPCFPFFLGVNPNVVAVLDVKSFAHLFDMEIVEKANVLVG

  121' DFRPSLGFYGGVRVGVYLDTTEPKHAKIKGFAMETLKRSSKVWLQELRSNLNIFWGTIES
  108* DFMPSVVYTGDMRVCAYLDTSEPKHTQIKNFSLDILKRSSKIWVPTLVKELDTLFGTFES

  181' EISKNGAASYIFPLQRCIFSFLCASLAGVDASVSPDIAENGWKTINTWLALQVIPTAKLG
  168* DLSKSKSASLLPALQKFLFNFFSLTFLGADPSASPEIANSGFAYLDAWLAIQLAPTVSIG

  241' VVPQPLEEILLHTWPYPSLLIAGNYKKLYNFIDENAGDCLRLGQEEFGLTRDEAIQNLLF
  228* VL-QPLEEIFVHSFSYPYFLVRGGYEKLIKFVKSEAKEVLTRAQTDFQLTEQEAIHNLLF

  301' VLGFNAYGGFSVFLPSLIGRITGD-NSGLQERIRTEVR-RVCGSGSDLNFKTVNEMELVK
  287* ILGFNAFGGFTIFLPTLLGNLGDEKNAEMQEKLRKEVREKVGTNQENLSFESVKEMELVQ

  359' SVVYETLRFSPPVPLQFARARKDFQISSHDAVFEVKKGELLCGYQPLVMRDANVFDEPEE
  347* SFVYESLRLSPPVPSQYARARKDFMLSSHDSVYEIKKGELLCGYQPLVMKDPKVFDEPEK

  419' FKPDRYVGETGSELLNYLYWSNGPQTGTPSASNKQCAAKDIVTLTASLLVADLFLRYDTI
  407* FMLERFTKEKGKELLNYLFWSNGPQTGSPTESNKQCAAKDAVTLTASLIVAYIFQKYDSV

  479' TGDSGSIKAVVKAK
  467* SFSSGSLTSVKKAC
```

Fig. 2

```
[ GENETYX : Amino Acid Sequence Homology Data ]

1st Amino Acid Sequence
  File Name         : Arabidopsis lyase AA
  Sequence Size     : 492

2nd Amino Acid Sequence
  File Name         : Arabidopsis AOS AA
  Sequence Size     : 517

  Unit Size to compare = 2



[39.2% / 485 aa]

    1'                                MLLRTMAATSPRPPPSTSLTSQQPPSPPSQLPLRTMPGS
                                      . .*... .  . ..*.  . ..... ...**.*..**.
    1* MASISTPFPISLHPKTVRSKPLKFRVLTRPIKASGSETPDLTVATRTGSKDLPIRNIPGN

   40' YGWPLVGPLSDRLDYFAFQGPDKFFRTRAEKYKSTVFRTNIPPTFPFFGNVNPNIVAVLD
       ** *.***..** ***. **...**..*  **.***.*.*.**. .*... **..**.**
   61* YGLPIVGPIKDRWDYFCDQGAEEFFKSRIRKYNSTVYRVNMPPG-AFIAE-NPQVVALLD

  100' VKSFSHLFDMDLVDKRDVLIGDFRPSLGFYGGVRVGVYLDTTEPKHAKIKGFAMETLKRS
        ***. ***.* *.*.*...*...** .. ** *.  ***..****.*.*... . **.*
  119* GKSFPVLFDVDKVEKGDLFTGTYMPSTELTGGYRILSYLDPSEPKHEKLKNLLFFLLKSS

  160' SKVWLQELRSNLNIFWGTIESEISKNGAASYIFPLQRCIFSFLCASLAGVDASVSPDIAE
       .. ..*.... . .....*.*          ....    .*  . **  .*.   *.* . . . .
  179* RNRIFPEFQATYSELFDSLEKEAFPLRESGFRRFQRRNRLLFLGSSFL-RDESRRYKLKA

  220' NGWKTINTWLALQVIPTAKLGVVPQPLEEILLHTWPYPSLLIAGNYKKLYNFIDENAGDC
       ..    *..*. ... * ..* .*. .** *.**.. *. *. ..*..**.*.    . *.
  238* DAPGLITKWVLFNLHPLLSIG-LPRVIEEPLIHTFSLPPALVKSDYQRLYEFLRIR-GEI

  280' LRLGQEEFGLTRDEAIQNLLFVLGFNAYGGFSVFLPSLIGRITGDNSGLQERIRTEVRRV
       * .....*..*.**..****. .**..**.....*... **. ..  ...*. .*.*.*
  296* L-VEADKLGISREEATQNLLFATSFNIWGGMKILFPNMVKRIGPGGHQVHNRLAEEIRSV

  340' C-GSGSDLNFKTVNEMELVKSVVYETLRFSPPVPLQFARARKDFQISSHDAVFEVKKGEL
         ..*..*.. ....***.****** *** *** *..**.**.  *.**.*.** **.
  355* IKSNGGELTMGAIEKMELTKSVVYECLRFEPPVTAQYGRAKKDLVIESHDAAFKVKAGEM

  399' LCGYQPLVMRDANVFDEPEEFKPDRYVGETGSELLNYLYWSNGPQTGTPSASNKQCAAKD
       *.*****. **...** ..** *.*.***.*..**... *****.*.**...*****.**
  415* LYGYQPLATRDPKIFDRADEFVPERFVGEEGEKLLRHVLWSNGPETETPTVGNKQCAGKD

  459' IVTLTASLLVADLFLRYDTITGDSGSIKAVVKAK
       .*.*.*.*.* ..* ***... . *.
  475* FVVLVARLFVIEIFRRYDSFDIEVGTSPLGSSVNFSSLRKASF
```

Fig. 3

[GENETYX: Translation of Nucleotide into Amino Acids]

1998.06.24

Filename : kmt15-1/full copy
Sequence Size : 1901
Sequence Position: 2 - 1901

```
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ttgataatgatattcagcttaccacccaacgccctcaaccttgactagccctccctcaaactctccttttttcaccaatctcactctccaaatagtatttt
       110        120        130        140        150        160        170        180        190        200
ctaggtttcatagtgataagcacatccacacaagagctatgaaatttctgaaacaaacgttacaatagacacacggcgtatttatttccctcaagcaatc
       210        220        230        240        250        260        270        280        290        300
gtatccccttagcagatcgaaaaaaaaagaagataccgataatgaattctgctcctctatcaactccggcgccggttactctgcccgtccgttcgattcc
                                          M  N  S  A  P  L  S  T  P  A  P  V  T  L  P  V  R  S  I  P
       310        320        330        340        350        360        370        380        390        400
aggcagctacggattgccgttagtagggccaatcgcggatcgattagactacttctggttccaaaaaccggaaaacttcttcaccaagagaatggaaaag
  G  S  Y  G  L  P  L  V  G  P  I  A  D  R  L  D  Y  F  W  F  Q  K  P  E  N  F  F  T  K  R  M  E  K
       410        420        430        440        450        460        470        480        490        500
cacaagagcacggtttttcagaacgaacgtgccgccgtgtttttccgtttttcggtagtgtgaatccaaatgtggtggcggttttagatgtgaaatcgtttt
  H  K  S  T  V  F  R  T  N  V  P  P  C  F  P  F  F  G  S  V  N  P  N  V  V  A  V  L  D  V  K  S  F  S
       510        520        530        540        550        560        570        580        590        600
cgcatctgtttgatatggagattgtagagaaagctaatgtgcttgttggggatttcatgcccagtgttgtttatactggagatatgcgtgttttgtgctta
    H  L  F  D  M  E  I  V  E  K  A  N  V  L  V  G  D  F  M  P  S  V  V  Y  T  G  D  M  R  V  C  A  Y
       610        620        630        640        650        660        670        680        690        700
tctcgatacttctgaacctaaacatgcccagattaagaatttttcacaggatattctaaaaagaggctcaaaaacatgggtgcctacactacttaaagag
  L  D  T  S  E  P  K  H  A  Q  I  K  N  F  S  Q  D  I  L  K  R  G  S  K  T  W  V  P  T  L  L  K  E
       710        720        730        740        750        760        770        780        790        800
cttgatacaatgtttacaacttttgaagcagatctttcaaaatccaatacagcttctcttcttcctgcactccaaaaattcctctttaacttcttctccc
  L  D  T  M  F  T  T  F  E  A  D  L  S  K  S  N  T  A  S  L  L  P  A  L  Q  K  F  L  F  N  F  F  S  L
       810        820        830        840        850        860        870        880        890        900
tcactatcctcggcgctgatccatctgtctccccagaaatcgccaattctggctacatcttttcttgattcatggctagctattcaattagcacctactgt
    T  I  L  G  A  D  P  S  V  S  P  E  I  A  N  S  G  Y  I  F  L  D  S  W  L  A  I  Q  L  A  P  T  V
       910        920        930        940        950        960        970        980        990       1000
cagcattggtgtccttcaacctcttgaagaaattttagtccattcttttgcataccctttttttcttgtcaaaggtaattatgaaaaactcgttcaattc
  S  I  G  V  L  Q  P  L  E  E  I  L  V  H  S  F  A  Y  P  F  F  L  V  K  G  N  Y  E  K  L  V  Q  F
      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
gtcaaaaatgaagccaaggaagttttaagcagggcacaaacagagtttcaactcacagaacaagaagccattcataacctttttgttcattcttgggttca
  V  K  N  E  A  K  E  V  L  S  R  A  Q  T  E  F  Q  L  T  E  Q  E  A  I  H  N  L  L  F  I  L  G  F  N
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
atgcttttggtggcttctccatttttcttgccaactcttttgggaaatcttggagatgagaaaaacgcggatatgcaagagaaactgagaaaagaagtgag
    A  F  G  G  F  S  I  F  L  P  T  L  L  G  N  L  G  D  E  K  N  A  D  M  Q  E  K  L  R  K  E  V  R
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
agacaaagtcggcgtaaatccagaaaacttgagttttgaaagtgttaaagaaatggaacttgttcagtcttttgtttatgaaacacttaggcttagtcca
  D  K  V  G  V  N  P  E  N  L  S  F  E  S  V  K  E  M  E  L  V  Q  S  F  V  Y  E  T  L  R  L  S  P
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
ccagtaccaagtcaatatgcaagagcaagaaaagattttaaactgagttcacatgattcagtttacgaaatcaagaaaggggagcttctttgtggttatc
  P  V  P  S  Q  Y  A  R  A  R  K  D  F  K  L  S  S  H  D  S  V  Y  E  I  K  K  G  E  L  L  C  G  Y  Q
```

Fig. 4A

```
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
agcctttagttatgaaagatccaaaggtgtttgatgaacctgaaaagtttgtgttggagagatttacaaaggaaaaagggaaagaattgctgaattattt
  P  L  V  M  K  D  P  K  V  F  D  E  P  E  K  F  V  L  E  R  F  T  K  E  K  G  K  E  L  L  N  Y  L

      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
gttttggtctaatggtccacaaactgggagacctactgaatcaaacaagcaatgtgctgctaaagatatggttactctaactgcttctttgattgtggct
 F  W  S  N  G  P  Q  T  G  R  P  T  E  S  N  K  Q  C  A  A  K  D  M  V  T  L  T  A  S  L  I  V  A

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
tacattttccaaaagtacgattctgtgagtttctcatctggttcactcacatctgtgaaaaaggctagttgagttctgggctgtgtagctgcaaaatcta
Y  I  F  Q  K  Y  D  S  V  S  F  S  S  G  S  L  T  S  V  K  K  A  S  *

      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
agcttgctagacatgttgatcttaagtctcggtggctggctttttccttcctccccctattgtgtaatataatttcacaaataaaagttcaacatgagtgt

      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900
acttattttctgttctgacttatgactattgtctatcctcttacaaattaagttggaacatgaatctaagttataattacaaaaaaaaaaaaaaaaaaaa
```

a

Fig. 4B

[GENETYX: Translation of Nucleotide into Amino Acids]

1998.06.24

Filename : C17/full copy
Sequence Size : 1712
Sequence Position: 1 - 1712

```
        10         20         30         40         50         60         70         80         90        100
ctcctcttctctctaccggaaaagttcaaccaacctctccccctcccttttcaaattcatactcccacatctctcaaatatcgatgcacctcaccatgacct
       110        120        130        140        150        160        170        180        190        200
ccggcggaatgccttccataccttcatctatttcccccccgccggtctctttacctctcagaaatatccccggcagctacggtttgccgctgctgggatc
       210        220        230        240        250        260        270        280        290        300
catcggtgaccgactcgattactactggtttcaaggacccgataagttcttcaggactcgtatggagaagaatcgaagcacggttttcagaaccaatgtt
       310        320        330        340        350        360        370        380        390        400
cctccgtctttccctttcatctccgctgatcccagagtagttgcggttctggattgcaagtcctttgcgcatctatttgacatggaaatcgtggagaaaa
       410        420        430        440        450        460        470        480        490        500
ataatgttctggtcggtgatttcatgcccagcatcagtttcaccggaaacatgagagtgtgtgcttatttggatacctcggaacccaatcactccaaggt
       510        520        530        540        550        560        570        580        590        600
aaagaacttcattacagacattctacggcggagctcaaggatatggatatcggagttggaatccaacctatcgacgatgtgggacggcattgaattagaa
       610        620        630        640        650        660        670        680        690        700
atggcaaagaacaaacaatcgggtttcagaaactttctccaaccagctcttttcaatttcttctccaaaactctcgccggcgccgacactgcaaaatcac
       710        720        730        740        750        760        770        780        790        800
cggaagtggcaaaatccggatacatagacgtcataatctggctcggcctccagctcgttcccaccatccacatcggcgttctccagccccttgaagaaat
       810        820        830        840        850        860        870        880        890        900
attccttcactctttccgcttacccttttccccattgcttcccgctaccaaagactctacgatttcttccaaaaagaaggggcagaagttgttgagcga
       910        920        930        940        950        960        970        980        990       1000
ggtgtcaccgagttcgggttaacaaaggaagaagccattcacaatctcatcttcaccatgggcttcaacgcctatggtggcttcagtctcttcttccctg
      1010       1020       1030       1040       1050       1060       1070       1080       1090       1100
ttctacttgaccgtatactcaatgataaaaccggtctacaacaaagaatcctcaaggaagtcaggtcaaaatccagctttggtctgaccttcgagtcagt
      1110       1120       1130       1140       1150       1160       1170       1180       1190       1200
caaggagatggacctcatctactccatcgtctacgagacactccggcttgacccccagttccctcccaatatgccagagccagaagatttcaagctaac
      1210       1220       1230       1240       1250       1260       1270       1280       1290       1300
ttcctacaattcaacgtataacatcaagaaaggggaactgctttgtgggtatcagccgctggtgatgagagatccagaggtgttcgatgaaccggaggcg
      1310       1320       1330       1340       1350       1360       1370       1380       1390       1400
tttaatccagaccgattccggggagagaaaggggcagcgctgctggattatttatttggtcgaatgggccgcaaacggggacaccgagcgagaagaaca
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
aacagtgtgctgggaaggatttagtggtgctgacgggggtggtgtttgtggcttacatatttaaaaggtatgattcgattgctggggaaggaggttccat
      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
tacagcttttcaaagggctaattgaaaagtgaaagtgaattatatatacattatgtatcgtttattgagagatgcacttttcttgttcaatggcttcttt
      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
cttatgtatgagtttggagccaaaatgggaagatttggaatgaataaatcaataaaatcaagatttactttaccttgtaaaaaaaaaaaaaaaaaaaaaa
      1710
aaaaaaaaaaaa
```

Fig. 5

[GENETYX: Translation of Nucleotide into Amino Acids]

1998.06.24

Filename : C15 full/cDNA copy
Sequence Size : 1813
Sequence Position: 2 - 1813

```
       10        20        30        40        50        60        70        80        90       100
ccatttctcttccaacgtgaagataagaaacctttgtttacttttttgtacgatcacaggtcacagcaatggcttcttcctcccctgaacttcctctcaaa
                                                                  M  A  S  S  S  P  E  L  P  L  K

      110       120       130       140       150       160       170       180       190       200
cccattcccggtggctatggcttccccttcctcggtcccatcaaagaccgttacgattacttctatttccaaggtagagacgaattcttccgttcccgaa
P  I  P  G  G  Y  G  F  P  F  L  G  P  I  K  D  R  Y  D  Y  F  Y  F  Q  G  R  D  E  F  F  R  S  R  I

      210       220       230       240       250       260       270       280       290       300
ttaccaaatacaactccaccgtcttccacgccaacatgccaccgggccccttcatctcctccgattccagagtcgttgtcctcctcgatgccctcagttt
  T  K  Y  N  S  T  V  F  H  A  N  M  P  P  G  P  F  I  S  S  D  S  R  V  V  V  L  L  D  A  L  S  F

      310       320       330       340       350       360       370       380       390       400
tcccatcctcttcgacaccaccaaagtcgagaaacgcaacattctcgacggaacttacatgccctccttgtccttcaccggcggcattcgcacctgtgct
 P  I  L  F  D  T  T  K  V  E  K  R  N  I  L  D  G  T  Y  M  P  S  L  S  F  T  G  G  I  R  T  C  A

      410       420       430       440       450       460       470       480       490       500
tatttggacccatcggaaacagagcacactgttctcaaacgcctctttctctcctttctcgcttctcaccatgacaggttcatccctctgtttcgaagct
Y  L  D  P  S  E  T  E  H  T  V  L  K  R  L  F  L  S  F  L  A  S  H  H  D  R  F  I  P  L  F  R  S  S

      510       520       530       540       550       560       570       580       590       600
ccttgtctgagatgtttgttaagcttgaagataaactcgccgacaaaaataagatcgctgatttcaactcgattagtgatgccgtgtcgtttgattatgt
  L  S  E  M  F  V  K  L  E  D  K  L  A  D  K  N  K  I  A  D  F  N  S  I  S  D  A  V  S  F  D  Y  V

      610       620       630       640       650       660       670       680       690       700
tttccgtttattctccgatggaacccctgattcgacattagctgctgatggacctggaatgttcgatttatggcttgggcttcaacttgccccattggct
 F  R  L  F  S  D  G  T  P  D  S  T  L  A  A  D  G  P  G  M  F  D  L  W  L  G  L  Q  L  A  P  L  A

      710       720       730       740       750       760       770       780       790       800
tccattggccttcccaaaattttctctgtttttgaagatctcattattcacaccattcccctgccttttcttcccagtcaagagtcgttacaggaagcttt
S  I  G  L  P  K  I  F  S  V  F  E  D  L  I  I  H  T  I  P  L  P  F  F  P  V  K  S  R  Y  R  K  L  Y

      810       820       830       840       850       860       870       880       890       900
ataaagcgttttactcctcctctggctcatttctagacgaagcagagaaacaggggatagacagagagaaagcatgtcacaatttagtgtttcttgctgg
  K  A  F  Y  S  S  S  G  S  F  L  D  E  A  E  K  Q  G  I  D  R  E  K  A  C  H  N  L  V  F  L  A  G

      910       920       930       940       950       960       970       980       990      1000
attcaacgcatacgggggaatgaaagtccttttttcccactatactgaaatgggtcggcaccggtggcgaggatctccaccgtaaactggcggaggaagtg
 F  N  A  Y  G  G  M  K  V  L  F  P  T  I  L  K  W  V  G  T  G  G  E  D  L  H  R  K  L  A  E  E  V

     1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
aggacaaccgtgaaggaagaaggggactgactttctccgccttggagaaaatgagtctgctgaagtcggtcgtgtacgaagctctgaggatcgaaccgc
R  T  T  V  K  E  E  G  G  L  T  F  S  A  L  E  K  M  S  L  L  K  S  V  V  Y  E  A  L  R  I  E  P  P

     1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
cggtgccgttccagtacgggaaagcgaaggaggatatcgtgattcagagccacgattcttgtttcaagatcaaaaaaggggagacgatttttggttatca
  V  P  F  Q  Y  G  K  A  K  E  D  I  V  I  Q  S  H  D  S  C  F  K  I  K  K  G  E  T  I  F  G  Y  Q

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
gccgtttgctactaaagatccgaagatttttaaggactcggagaagttcgtgggcgataggttcgtgggagaagaaggggagaagcttttgaagtatgtt
 P  F  A  T  K  D  P  K  I  F  K  D  S  E  K  F  V  G  D  R  F  V  G  E  E  G  E  K  L  L  K  Y  V

     1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
tactggtcaaatgagcgggagacggtggagccgacggcggagaacaagcagtgtccggggaagaatctggtggtgatgatgggcaggattattgtggtgg
Y  W  S  N  E  R  E  T  V  E  P  T  A  E  N  K  Q  C  P  G  K  N  L  V  V  M  M  G  R  I  I  V  V  E
```

Fig. 6A

```
      1410       1420       1430       1440       1450       1460       1470       1480       1490       1500
aattcttcctccgttatgatacgttcactgtggacgtcgcagatttggcgctgggcccggcggtgaagttcaagtccttgaccagagccaccgcttcggt
  F   F   L   R   Y   D   T   F   T   V   D   V   A   D   L   A   L   G   P   A   V   K   F   K   S   L   T   R   A   T   A   S   V

      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
ttagaagctaatgacaaaattagtttttaatcattttacagaataaaggaagggtgttcgtgattggttgatgcagggaagctgtggtggacttgagcac
 *

      1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
acatgacacatcattgatttgttggggttataaagtaatttcctcgtgatccgcgtcgtttttaatttgagatctcattgtgtgttgtaacccgcgggtg

      1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
atcttatttttatagtttctttttttctcaactatgctccaatttttaaattaaataaataccaaatatcaacttcatagacaaaaaaaaaaaaaaaaaa

      1810
aaaaaaaaaaaaa
```

Fig. 6B

```
CLUSTAL W (1.7) Multiple Sequence Alignments


Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: gi|1272340       480 aa
Sequence 2: banana           483 aa
Sequence 3: ARABIDOPSIS      492 aa
Start of Pairwise alignments
Aligning...
Sequences (1:2) Aligned. Score:  51
Sequences (1:3) Aligned. Score:  55
Sequences (2:3) Aligned. Score:  53
Start of Multiple Alignment
There are 2 groups
Aligning...
Group 1: Sequences:   2      Score:4874
Group 2: Sequences:   3      Score:4967
Alignment Score 5148
CLUSTAL-Alignment file created  [clustalw.aln]
CLUSTAL W (1.7) multiple sequence alignment


gi|1272340       ---------------MIPIMSSAPLSTATPISLPVRKIPGSYGFPLLGPLWDRLDYNWFQ
banana           ---------------MAMMWSSASATAVT--TLPTRPIPGSYGPPLVGPLKDRLDYFWFQ
ARABIDOPSIS      MLLRTMAATSPRPPPSTSLTSQQPPSPPS--QLPLRTMPGSYGWPLVGPLSDRLDYFWFQ
                                 : *. . :. :   ** * :***** **:*** ***** ***

gi|1272340       KLPDFFSKRVEKYNSTVFRTNVPPCFPFFLGVNPNVVAVLDVKSFAHLFDMEIVEKANVL
banana           GPETFFRSRMATHKSTVFRTNMPPTFPFFVGVDPRVVTVLDCTSFSALFDLQVVEKKNIL
ARABIDOPSIS      GPDKFFRTRAEKYKSTVFRTNIPPTFPFFGNVNPNIVAVLDVKSFSHLFDMDLVDKRDVL
                     ** .*  .::*******:** **** .*:*.:*:*** .**: ***:::*:* ::*

gi|1272340       VGDFMPSVVYTGDMRVCAYLDTSEPKHTQIKNFSLDILKRSSKTWVPTLVKELDTLFGTF
banana           IGDYMPSLSFTGDTRVVVYLDPSEPDHARVKSFCLQLLRRGAKTWVSSFLSNLDVMLATI
ARABIDOPSIS      IGDFRPSLGFYGGVRVGVYLDTTEPKHAKIKGFAMETLKRSSKVWLQELRSNLNIFWGTI
                 :**: **: : *. ** .***.:**.*:::*.*.:: *:*.:*.*:  : .:*: : .*:

gi|1272340       ESDLSKSKSASLLPALQKFLFNFFSLTFLGADPSASPEIANSGFAYLDAWLAIQLAPTVS
banana           EQGIAKDGSAGLFGPLQKCIFAFLCKSIIGADPSVSPDVGENGFVMLDKWLALQLLPTVK
ARABIDOPSIS      ESEISKNGAASYIFPLQRCIFSFLCASLAGVDASVSPDIAENGWKTINTWLALQVIPTAK
                 *. ::*. :*. : .**: :* *:. :: *.*.*.**::.:.*:  :: ***:*: **..

gi|1272340       IG-VLQPLEEIFVHSFSYPYFLVRGGYEKLIKFVKSEAKEVLTRAQTDFQLTEQEAIHNL
banana           VGAIPQPLEEILLHSFPLPFFLVSRDYRKLYEFVEKQGQEVVRRAETEHGLSKHDAINNI
ARABIDOPSIS      LGVVPQPLEEILLHTWPYPSLLIAGNYKKLYNFIDENAGDCLRLGQEEFGLTRDEAIQNL
                 :* : ******::*::. * :*:  .*.** :*:..:. : :  .: :. *:..:**:*:

gi|1272340       LFILGFNAFGGFTIFLPTLLGNLGDEKNAEMQEKLRKEVREKVGTNQEN-LSFESVKEME
banana           LFVLGFNAFGGFSVFFPTLLTTIGRDK-TGLREKLKDEVRRVMKSRGEKRPSFETVREME
ARABIDOPSIS      LFVLGFNAYGGFSVFLPSLIGRITGDN-SGLQERIRTEVRRVCGS-GSD-LNFKTVNEME
                 **:*****:***::*:*:*:.  :  :: : ::*::: ***.   :  ..  .*::*.***

gi|1272340       LVQSFVYESLRLSPPVPSQYARARKDFMLSSHDSVYEIKKGELLCGYQPLVMKDPKVFDE
banana           LVRSTVYEVLRLNPPVPLQUGRARTDFTLNSHDAAFKVEKGELLCGYQPLVMRDPAVFDD
ARABIDOPSIS      LVKSVVYETLRFSPPVPLQFARARKDFQISSHDAVFEVKKGELLCGYQPLVMRDANVFDE
                 **:* *** **:.**** * .***.** :.***:.:::::************:*. ***:*

gi|1272340       PEKFMLERFTKEKGKELLNYLFWSNGPQTGSPTESNKQCAAKDAVTLTASLIVAYIFQKY
banana           PETFAPERFMGS-GKELLKYVFWSNGPETGTPTPANKQCAAKDYVVETACLLMAEIFYRY
ARABIDOPSIS      PEEFKPDRYVGETGSELLNYLYWSNGPQTGTPSASNKQCAAKDIVTLTASLLVADLFLRY
                 ** *  :*:  . *.***:*::*****:**:*: :******** *. **.*::* :* :*

gi|1272340       DSVSFSSGSLTSVKKAC-----
banana           DEFVCADDAISVTKLDRAREWE
ARABIDOPSIS      DTITGDSGSIKAVVKAK-----
                 * .   ..::. .
```

Fig. 7

1. nucleotide

	Ca HPOL	Le HPOL	C17	Al HPOL	Ms HPOL	C15	Li AOS	At AOS
Ca HPOL		84.6	59.9	59.7	55.0	50.0	52.4	52.6
Le HPOL	84.6		59.4	59.5	55.6	50.9	49.9	53.1
Cs17 HPOL	60.0	59.5		61.0	58.6	52.9	54.5	52.5
Al HPOL	59.7	59.5	61.0		58.3	52.0	52.1	51.7
Ms HPOL	55.1	55.6	58.7	58.3		54.1	56.4	51.7
Cs15 HPOL	50.0	50.7	52.8	52.1	53.9		58.1	57.6
Li AOS	52.1	49.7	54.5	52.3	56.5	58.3		61.8
At AOS	52.5	53.2	52.5	51.7	51.8	57.8	62.3	

Figure 8A

2. amino acid

	Ca HPOL	Le HPOL	C17	Al HPOL	Ms HPOL	C15	Gu AOS	Li AOS	At AOS
Ca HPOL		88.2	59.5	57.0	54.1	39.6	41.0	40.3	40.0
Le HPOL	88.2		61.4	58.7	57.3	39.4	41.6	40.5	40.4
Cs17 HPOL	59.5	61.4		56.2	53.6	41.7	43.3	41.9	41.1
Al HPOL	57.0	58.7	56.2		56.1	38.3	41.1	41.1	39.2
Ms HPOL	54.1	57.3	53.6	56.1		41.9	44.5	44.5	43.2
Cs15 HPOL	39.6	39.4	41.7	38.4	41.9		51.6	51.2	51.4
Gu AOS	41.0	41.6	43.3	41.1	44.5	51.6		65.9	60.3
Li AOS	40.3	40.5	41.9	41.1	44.5	51.2	65.9		58.2
At AOS	40.0	40.4	41.1	39.2	43.2	51.4	60.3	58.2	

Figure 8B

Figure 8

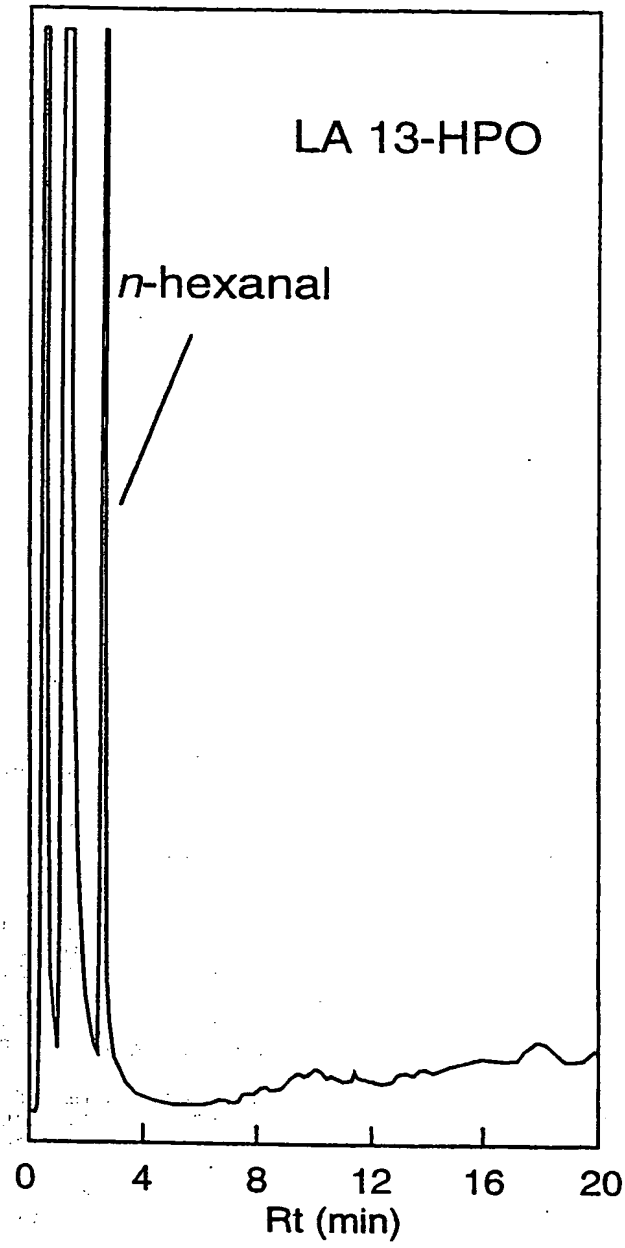

Figure 9A

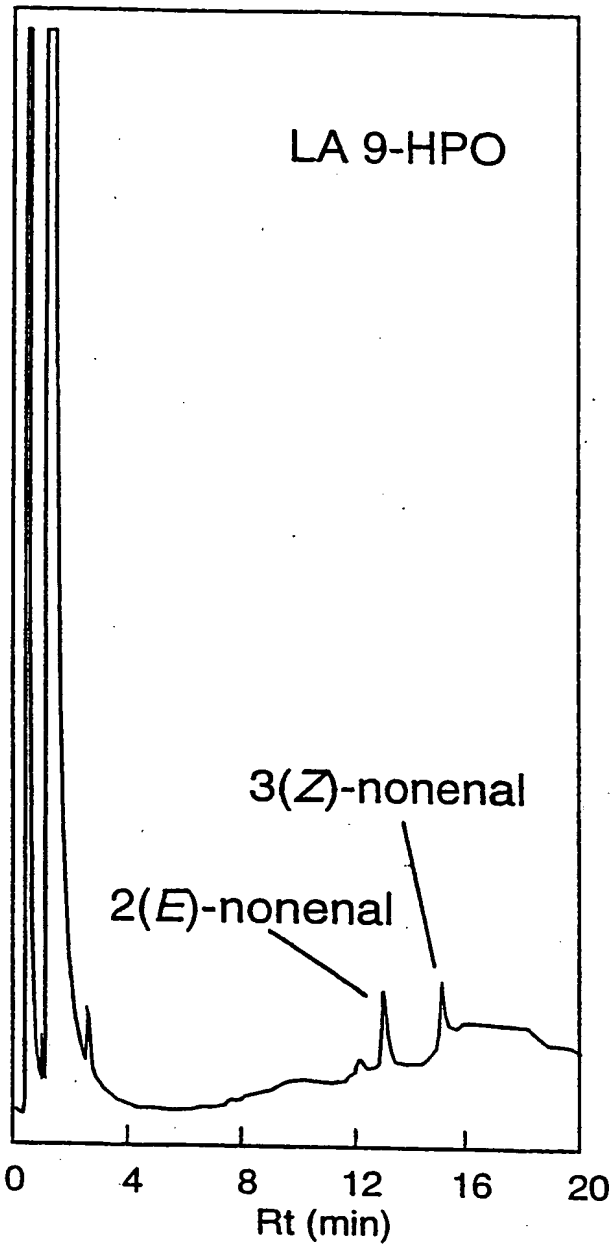

Figure 9B

Figure 9

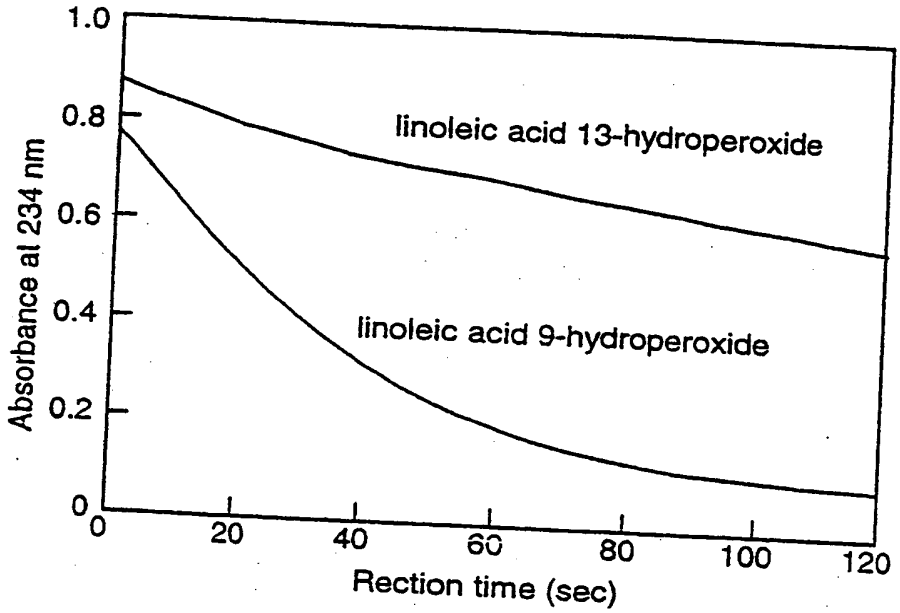

Figure 10

SEQUENCE LISTING

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        35                  40                  45
Ser Lys Asp Pro Ile Ser Phe Ser Gly Gln Glu Leu Arg Ser Ile Arg
    50                  55                  60
Ala Leu Cys Ser Val Gln Ile Phe Leu Arg Arg Phe Leu Ser Ser Ala
65                  70                  75                  80
Thr His Leu Phe Asp Met Asp Leu Val Asp Lys Arg Asp Val Leu Ile
                85                  90                  95
Gly Asp Phe Arg Pro Ser Leu Gly Phe Tyr Gly Gly Val Arg Val Gly
            100                 105                 110
Val Tyr Leu Asp Thr Thr Glu Pro Lys His Ala Lys Val Arg Thr Tyr
        115                 120                 125
Val Ala Leu Leu Leu Leu His Lys Arg Phe Arg Tyr Gly Asn Thr Lys
    130                 135                 140
Thr Lys Leu Lys Ser Met Ala Thr Arg Ala Ser Phe Lys Pro Lys His
145                 150                 155                 160
Phe Leu Gly Asn Asn Arg Ile Arg Asn Leu Gln Lys Arg Phe Leu Cys
                165                 170                 175
Ala Ser Leu Ala Gly Val Asp Asp Ala Ser Val Ser Pro Asp Ile Ala
            180                 185                 190
Glu Asn Gly Trp Lys Thr Ile Asn Thr Trp Leu Ala Leu Gln Val Ile
        195                 200                 205
Pro Thr Ala Lys Leu Gly Val Val Pro Gln Pro Leu Glu Glu Ile Leu
    210                 215                 220
Leu His Thr Trp Pro Tyr Pro Ser Leu Leu Ile Ala Gly Asn Tyr Lys
225                 230                 235                 240
Lys Leu Tyr Asn Phe Ile Asp Glu Asn Ala Gly Asp Cys Leu Arg Leu
                245                 250                 255
Gly Gln Glu Glu Phe Gly Leu Thr Arg Asp Glu Ala Ile Gln Asn Leu
            260                 265                 270
Leu Phe Val Leu Gly Phe Asn Ala Tyr Gly Gly Phe Ser Val Phe Leu
        275                 280                 285
Pro Ser Leu Ile Gly Arg Ile Thr Gly Asp Asn Ser Gly Leu Gln Glu
    290                 295                 300
Arg Ile Arg Thr Glu Val Arg Arg Val Cys Gly Ser Gly Ser Asp Leu
305                 310                 315                 320
Asn Phe Lys Thr Val Asn Glu Met Glu Leu Val Lys Ser Val Val Tyr
                325                 330                 335
Glu Thr Leu Arg Phe Ser Pro Pro Val Pro Leu Gln Phe Ala Arg Ala
            340                 345                 350
Arg Lys Asp Phe Gln Ile Ser Ser His Asp Ala Val Phe Glu Val Lys
        355                 360                 365
Lys Gly Glu Leu Leu Cys Gly Tyr Gln Pro Leu Val Met Arg Asp Ala
    370                 375                 380
Asn Val Phe Asp Glu Pro Glu Glu Phe Lys Pro Asp Arg Tyr Val Gly
385                 390                 395                 400
Glu Thr Gly Ser Glu Leu Leu Asn Tyr Leu Tyr Trp Ser Asn Gly Pro
                405                 410                 415
Gln Thr Gly Thr Pro Ser Ala Ser Asn Lys Gln Cys Ala Ala Lys Asp
            420                 425                 430
Ile Val Thr Leu Thr Ala Ser Leu Leu Val Ala Asp Leu Phe Leu Arg
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Ser Asp Arg Leu Asp Tyr Phe Trp Phe Gln Gly Pro Asp Lys Phe Phe
    50                  55                  60
Arg Thr Arg Ala Glu Lys Tyr Lys Ser Thr Val Phe Arg Thr Asn Ile
65                  70                  75                  80
Pro Pro Thr Phe Pro Phe Phe Gly Asn Val Asn Pro Asn Ile Val Ala
                85                  90                  95
Val Leu Asp Val Lys Ser Phe Ser His Leu Phe Asp Met Asp Leu Val
            100                 105                 110
Asp Lys Arg Asp Val Leu Ile Gly Asp Phe Arg Pro Ser Leu Gly Phe
        115                 120                 125
Tyr Gly Gly Val Arg Val Gly Val Tyr Leu Asp Thr Thr Glu Pro Lys
    130                 135                 140
His Ala Lys Ile Lys Gly Phe Ala Met Glu Thr Leu Lys Arg Ser Ser
145                 150                 155                 160
Lys Val Trp Leu Gln Glu Leu Arg Ser Asn Leu Asn Ile Phe Trp Gly
                165                 170                 175
Thr Ile Glu Ser Glu Ile Ser Lys Asn Gly Ala Ala Ser Tyr Ile Phe
            180                 185                 190
Pro Leu Gln Arg Cys Ile Phe Ser Phe Leu Cys Ala Ser Leu Ala Gly
        195                 200                 205
Val Asp Ala Ser Val Ser Pro Asp Ile Ala Glu Asn Gly Trp Lys Thr
    210                 215                 220
Ile Asn Thr Trp Leu Ala Leu Gln Val Ile Pro Thr Ala Lys Leu Gly
225                 230                 235                 240
Val Val Pro Gln Pro Leu Glu Glu Ile Leu Leu His Thr Trp Pro Tyr
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Pro Ser Leu Leu Ile Ala Gly Asn Tyr Lys Lys Leu Tyr Asn Phe Ile
            260                 265                 270
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Leu Thr Arg Asp Glu Ala Ile Gln Asn Leu Leu Phe Val Leu Gly Phe
    290                 295                 300
Asn Ala Tyr Gly Gly Phe Ser Val Phe Leu Pro Ser Leu Ile Gly Arg
305                 310                 315                 320
Ile Thr Gly Asp Asn Ser Gly Leu Gln Glu Arg Ile Arg Thr Glu Val
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Arg Arg Val Cys Gly Ser Gly Ser Asp Leu Asn Phe Lys Thr Val Asn
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Glu Met Glu Leu Val Lys Ser Val Val Tyr Glu Thr Leu Arg Phe Ser
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385                 390                 395                 400
Gly Tyr Gln Pro Leu Val Met Arg Asp Ala Asn Val Phe Asp Glu Pro
                405                 410                 415
Glu Glu Phe Lys Pro Asp Arg Tyr Val Gly Glu Thr Gly Ser Glu Leu
            420                 425                 430
Leu Asn Tyr Leu Tyr Trp Ser Asn Gly Pro Gln Thr Gly Thr Pro Ser
        435                 440                 445
Ala Ser Asn Lys Gln Cys Ala Ala Lys Asp Ile Val Thr Leu Thr Ala
    450                 455                 460
Ser Leu Leu Val Ala Asp Leu Phe Leu Arg Tyr Asp Thr Ile Thr Gly
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        35                  40                  45
Asp Phe Phe Ser Lys Arg Val Glu Lys Tyr Asn Ser Thr Val Phe Arg
    50                  55                  60
Thr Asn Val Pro Pro Cys Phe Pro Phe Phe Leu Gly Val Asn Pro Asn
65                  70                  75                  80
Val Val Ala Val Leu Asp Val Lys Ser Phe Ala His Leu Phe Asp Met
                85                  90                  95
Glu Ile Val Glu Lys Ala Asn Val Leu Val Gly Asp Phe Met Pro Ser
            100                 105                 110
Val Val Tyr Thr Gly Asp Met Arg Val Cys Ala Tyr Leu Asp Thr Ser
        115                 120                 125
Glu Pro Lys His Thr Gln Ile Lys Asn Phe Ser Leu Asp Ile Leu Lys
    130                 135                 140
Arg Ser Ser Lys Thr Trp Val Pro Thr Leu Val Lys Glu Leu Asp Thr
145                 150                 155                 160
Leu Phe Gly Thr Phe Glu Ser Asp Leu Ser Lys Ser Lys Ser Ala Ser
                165                 170                 175
Leu Leu Pro Ala Leu Gln Lys Phe Leu Phe Asn Phe Phe Ser Leu Thr
            180                 185                 190
Phe Leu Gly Ala Asp Pro Ser Ala Ser Pro Glu Ile Ala Asn Ser Gly
        195                 200                 205
Phe Ala Tyr Leu Asp Ala Trp Leu Ala Ile Gln Leu Ala Pro Thr Val
    210                 215                 220
Ser Ile Gly Val Leu Gln Pro Leu Glu Glu Ile Phe Val His Ser Phe
225                 230                 235                 240
Ser Tyr Pro Tyr Phe Leu Val Arg Gly Gly Tyr Glu Lys Leu Ile Lys
                245                 250                 255
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Phe Gln Leu Thr Glu Gln Glu Ala Ile His Asn Leu Leu Phe Ile Leu
        275                 280                 285
Gly Phe Asn Ala Phe Gly Gly Phe Thr Ile Phe Leu Pro Thr Leu Leu
    290                 295                 300
Gly Asn Leu Gly Asp Glu Lys Asn Ala Glu Met Gln Glu Lys Leu Arg
305                 310                 315                 320
Lys Glu Val Arg Glu Lys Val Gly Thr Asn Gln Glu Asn Leu Ser Phe
                325                 330                 335
Glu Ser Val Lys Glu Met Glu Leu Val Gln Ser Phe Val Tyr Glu Ser
            340                 345                 350
Leu Arg Leu Ser Pro Pro Val Pro Ser Gln Tyr Ala Arg Ala Arg Lys
        355                 360                 365
Asp Phe Met Leu Ser Ser His Asp Ser Val Tyr Glu Ile Lys Lys Gly
    370                 375                 380
Glu Leu Leu Cys Gly Tyr Gln Pro Leu Val Met Lys Asp Pro Lys Val
385                 390                 395                 400
Phe Asp Glu Pro Glu Lys Phe Met Leu Glu Arg Phe Thr Lys Glu Lys
                405                 410                 415
Gly Lys Glu Leu Leu Asn Tyr Leu Phe Trp Ser Asn Gly Pro Gln Thr
            420                 425                 430
Gly Ser Pro Thr Glu Ser Asn Lys Gln Cys Ala Ala Lys Asp Ala Val
        435                 440                 445
Thr Leu Thr Ala Ser Leu Ile Val Ala Tyr Ile Phe Gln Lys Tyr Asp
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        35                  40                  45
Ser Lys Asp Leu Pro Ile Arg Asn Ile Pro Gly Asn Tyr Gly Leu Pro
    50                  55                  60
Ile Val Gly Pro Ile Lys Asp Arg Trp Asp Tyr Phe Tyr Asp Gln Gly
65                  70                  75                  80
Ala Glu Glu Phe Phe Lys Ser Arg Ile Arg Lys Tyr Asn Ser Thr Val
                85                  90                  95
Tyr Arg Val Asn Met Pro Pro Gly Ala Phe Ile Ala Glu Asn Pro Gln
            100                 105                 110
Val Val Ala Leu Leu Asp Gly Lys Ser Phe Pro Val Leu Phe Asp Val
        115                 120                 125
Asp Lys Val Glu Lys Lys Asp Leu Phe Thr Gly Thr Tyr Met Pro Ser
    130                 135                 140
Thr Glu Leu Thr Gly Gly Tyr Arg Ile Leu Ser Tyr Leu Asp Pro Ser
145                 150                 155                 160
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Glu Pro Lys His Glu Lys Leu Lys Asn Leu Leu Phe Phe Leu Leu Lys
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180 185 190
Leu Phe Asp Ser Leu Glu Lys Glu Ala Phe Pro Leu Arg Glu Ser Gly
195 200 205
Phe Arg Arg Phe Gln Arg Arg Asn Arg Leu Leu Phe Leu Gly Ser Ser
210 215 220
Phe Leu Arg Asp Glu Ser Arg Arg Tyr Lys Leu Lys Ala Asp Ala Pro
225 230 235 240
Gly Leu Ile Thr Lys Trp Val Leu Phe Asn Leu His Pro Leu Leu Ser
245 250 255
Thr Gly Leu Pro Arg Val Ile Glu Glu Pro Leu Ile His Thr Phe Ser
260 265 270
Leu Pro Pro Ala Leu Val Lys Ser Asp Tyr Gln Arg Leu Tyr Glu Phe
275 280 285
Leu Arg Ile Arg Gly Glu Ile Leu Val Glu Ala Asp Lys Leu Gly Ile
290 295 300
Ser Arg Glu Glu Ala Thr His Asn Leu Leu Phe Ala Thr Ser Phe Asn
305 310 315 320
Thr Trp Gly Gly Met Lys Ile Leu Phe Pro Asn Met Val Lys Arg Ile
325 330 335
Gly Pro Gly Gly His Gln Val His Asn Arg Leu Ala Glu Glu Ile Arg
340 345 350
Ser Val Ile Lys Ser Asn Gly Gly Glu Leu Thr Met Gly Ala Ile Glu
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Lys Met Glu Leu Thr Lys Ser Val Val Tyr Glu Cys Leu Arg Phe Glu
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Pro Pro Val Thr Ala Gln Tyr Gly Arg Ala Lys Lys Asp Leu Val Ile
385 390 395 400
Glu Ser His Asp Ala Ala Phe Lys Val Lys Ala Gly Glu Met Leu Tyr
405 410 415
Gly Tyr Gln Pro Leu Ala Thr Arg Asp Pro Lys Ile Phe Asp Arg Ala
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Arg Lys Ala Ser Phe
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aatgaattct gctcctctat caactccggc gccggttact ctgcccgtcc gttcgattcc     300
gggcagctac ggattgccgt tagtagggcc aatcgcggat cgattagact acttctggtt     360
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Asp Arg Leu Asp Tyr Phe Trp Phe Gln Lys Pro Glu Asn Phe Phe Thr
        35                  40                  45
Lys Arg Met Glu Lys His Lys Ser Thr Val Phe Arg Thr Asn Val Pro
    50                  55                  60
Pro Val Phe Pro Phe Phe Gly Ser Val Asn Pro Asn Val Val Ala Val
65                  70                  75                  80
Leu Asp Val Lys Ser Phe Ser His Leu Phe Asp Met Glu Ile Val Glu
                85                  90                  95
Lys Ala Asn Val Leu Val Gly Asp Phe Met Pro Ser Val Val Tyr Thr
            100                 105                 110
Gly Asp Met Arg Val Cys Ala Tyr Leu Asp Thr Ser Glu Pro Lys His
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        115                 120                 125
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145                 150                 155                 160
Phe Glu Ala Asp Leu Ser Lys Ser Asn Thr Ala Ser Leu Leu Pro Ala
                165                 170                 175
Leu Gln Lys Phe Leu Phe Asn Phe Phe Ser Leu Thr Ile Leu Gly Ala
            180                 185                 190
Asp Pro Ser Val Ser Pro Glu Ile Ala Asn Ser Gly Tyr Ile Phe Leu
        195                 200                 205
Asp Ser Trp Leu Ala Ile Gln Leu Ala Pro Thr Val Ser Ile Gly Val
    210                 215                 220
Leu Gln Pro Leu Glu Glu Ile Leu Val His Ser Phe Ala Tyr Pro Phe
225                 230                 235                 240
Phe Leu Val Lys Gly Asn Tyr Glu Lys Leu Val Gln Phe Val Lys Asn
                245                 250                 255
Glu Ala Lys Glu Val Leu Ser Arg Ala Gln Thr Glu Phe Gln Leu Thr
            260                 265                 270
Glu Gln Glu Ala Ile His Asn Leu Leu Phe Ile Leu Gly Phe Asn Ala
        275                 280                 285
Phe Gly Gly Phe Ser Ile Phe Leu Pro Thr Leu Leu Gly Asn Leu Gly
    290                 295                 300
Asp Glu Lys Asn Ala Asp Met Gln Glu Lys Leu Arg Lys Glu Val Arg
305                 310                 315                 320
Asp Lys Val Gly Val Asn Pro Glu Asn Leu Ser Phe Glu Ser Val Lys
                325                 330                 335
Glu Met Glu Leu Val Gln Ser Phe Val Tyr Glu Thr Leu Arg Leu Ser
            340                 345                 350
Pro Pro Val Pro Ser Gln Tyr Ala Arg Ala Arg Lys Asp Phe Lys Leu
        355                 360                 365
Ser Ser His Asp Ser Val Tyr Glu Ile Lys Lys Gly Glu Leu Leu Cys
    370                 375                 380
Gly Tyr Gln Pro Leu Val Met Lys Asp Pro Lys Val Phe Asp Glu Pro
385                 390                 395                 400
Glu Lys Phe Val Leu Glu Arg Phe Thr Lys Glu Lys Gly Lys Glu Leu
                405                 410                 415
Leu Asn Tyr Leu Phe Trp Ser Asn Gly Pro Gln Thr Gly Arg Pro Thr
            420                 425                 430
Glu Ser Asn Lys Gln Cys Ala Ala Lys Asp Met Val Thr Leu Thr Ala
        435                 440                 445
Ser Leu Ile Val Ala Tyr Ile Phe Gln Lys Tyr Asp Ser Val Ser Phe
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Ser Ser Gly Ser Leu Thr Ser Val Lys Lys Ala Ser
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cctccgtctt tccctttcat ctccgctgat cccagagtag ttgcggttct ggattgcaag 360
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International Bureau

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(30) Priority Data:
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(72) Inventor: MATSUI, Kenji; 2-51, Nishiki-cho, Yamaguchi City, Yamaguchi 753-0068 (JP).

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(81) Designated States *(national)*: CA, CN, JP, MX.

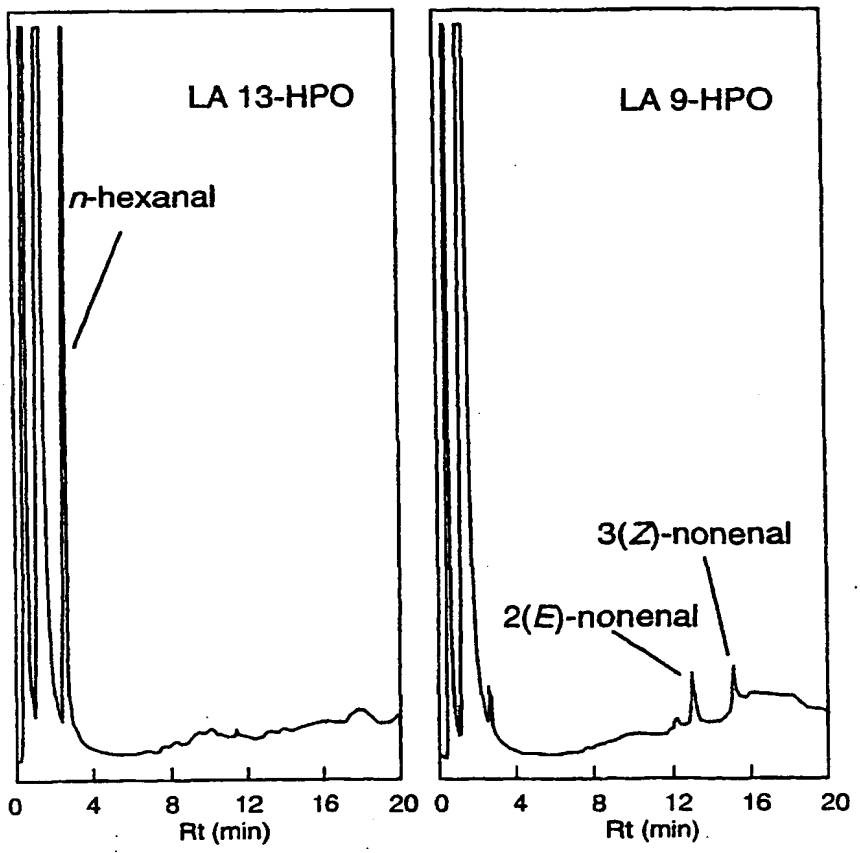
(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *with international search report*

(88) Date of publication of the international search report:
13 September 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NUCLEIC ACID SEQUENCE OF A CUCUMBER (CUCUMIS SATIVUS) FATTY ACID 9-HYDROPEROXIDE LYASE



(57) Abstract: This invention relates to 9-Hydroperoxide Lyase or 9-HPO lyase enzymes. DNA constructs useful for the expression of a plant HPO lyase in a cell are described. Furthermore, DNA constructs useful for the antisense expression of a 9-HPO lyase in a plant cell are described. Such constructs will contain a DNA sequence encoding the 9-HPO lyase of interest under the control of regulatory elements capable of preferentially directing the expression of the 9-HPO lyase in plant tissue, when such a construct is expressed in a trangenic plant. This invention also relates to methods of using a DNA sequence encoding a 9-HPO lyase for the modification of the volatile aldehydes in plant tissues, as well as for methods of increasing disease resistance in a plant. 9-HPO Lyase sequences exemplified herein are obtained from *Arabidopsis*.

WO 00/50575 A3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/05311

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/88 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, EMBASE, SCISEARCH, BIOTECHNOLOGY ABS, CHEM ABS Data, CAB Data, GENSEQ, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MATSUI K ET AL: "SEPARATION OF 13 AND 9 HYDROPEROXIDE LYASE ACTIVITIES IN COTYLEDONS OF CUCUMBER SEEDLINGS" ZEITSCHRIFT FUER NATURFORSCHUNG SECTION C BIOSCIENCES, vol. 44, no. 9-10, 1989, pages 883-885, XP000949968 ISSN: 0341-0382 the whole document --- -/--	1-11

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 2000	23/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

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